INTRODUCING

The Micro-Production Unit

MPU

a versatile, economic system
for the production of
high-quality legume Inoculants

A PLANNING GUIDE

NifTAL Center
1000 Holomua Road
Paia, Hawaii 96779 USA

phone 808-579-9568
fax 808-579-8516

Preface

This booklet provides an introduction to the Micro-Production Unit (MPU) for small-scale production of legume inoculants. It is intended to be planning guide, and not an instructional manual.

The MPU system is flexible -- it can be used effectively under a wide range of conditions and with minimal investment. This booklet offers suggestions for facilities design and equipment options for a relatively sophisticated production enterprise. Actual requirements will vary with site, scale of production, and conditions in existing facilities.

Acknowledgements

The MPU is based on research by NifTAL microbiologist Padma Somasegaran (1982 and 1995, reprints attached). This booklet, tables, and graphics were developed by NifTAL inoculant production specialist, Joseph Rourke.

The University of Hawaii NifTAL Center receives core funding from the US Agency for International Development through the Soil Management Collaborative Research Support Program (TropSoils CRSP) to develop and transfer biological nitrogen fixation technologies for sustainable agriculture.

Draft
26 April, 1993

The Micro-Production Unit (MPU)

The use of inoculants containing rhizobia, a nitrogen-fixing soil bacteria, is a proven biotechnology for enhancing legume production. In many areas, inoculants have substantially increased legume crop yields by maximizing the BNF process. NifTAL's goal is to make effective, low-cost BNF technologies and products available to farmers in developing countries. NifTAL combines applied research, product development, training, technical assistance, and support of commercial enterprises to achieve this goal.

One NifTAL emphasis has been the development and delivery of appropriate technologies for the small-scale production of rhizobial inoculants. The result of this effort is the Micro-Production Unit (MPU). The MPU relies on two key components: the use of a sterile inoculant carrier such as peat, and the dilution of pure broth cultures of rhizobia. By integrating these components, cost-efficient, decentralized, small-scale commercial inoculant production is now possible.

Advantages of the MPU. There are several advantages to producing rhizobial inoculants with the MPU compared to conventional large-scale commercial production methods. These include

* Initial investment costs are reduced. (See Table 1. Comparison of costs and operations time for sterile and non-sterile production modes.)

* The number and size of fermentor vessels for culturing rhizobia are reduced with the dilution technique. (See Table 2. Fermentor capacities and operations time to meet production goals.) Diluting the broth culture increases potential production capability by the amount of the dilution factor.

* High quality inoculants can be produced by diluting broth cultures up to **1000 fold** before mixing in sterile peat carrier. See Appendix for articles by Somasegaran for details.

* Production schedules are flexible. Producers can respond to demands for varying inoculant quantities and for a greater variety of specific inoculants.

* The use of a sterile carrier increases the shelf-life of inoculants. Over time (6-9 months), the quality of the sterile peat-based inoculants will be higher than non-sterile inoculants stored under similar conditions.

* Storage of sterile peat-based inoculants require less stringent conditions. Refrigerated storage is not as critical for maintaining inoculant quality.
* The MPU can easily be established in rural locations close to legume production areas, and as a result, distribution costs are reduced.
* Increasing the production capacity of the MPU is easy and inexpensive. (See Tables 1 and 2. See also Table 3 Potential production capacities of sterile and non-sterile production modes, and Table 4. Time requirements for injecting peat bags.) Note : These worksheets are interactive spreadsheets on computer. See Appendix for further details.
* Expanding the scale of production is easy and inexpensive. Fermentors used for small-scale operations will not become obsolete. Low-volume fermentors may be kept for the production of "custom" inoculants, late requests, or "starter" cultures for larger fermentors. Quality control procedures are simpler and more reliable. Because of the sterile carrier, rapid, direct plating of inoculants will provide accurate assessments of inoculant quality.

Conditions for Success. There are several conditions for successful inoculant production with the MPU systems.

* The dilution technique requires a reliable source of sterile pre packaged peat. There are reliable international sources of this peat if local sources are not available, but potential problems involving importation, transport, and foreign exchange must be considered.
* Some specialized equipment is needed, such as a sterilizable, adjustable injection pump system that can deliver set volumes of diluted cultured broth.
* The system requires a means of sterilizing large quantities of water for diluting the broth culture.
  Sterile peat bags must be injected individually. Mixing large batches of sterile peat and diluted broth is not economically feasible.
* Aseptic conditions must be maintained during production.
* A trained technical person is needed for certain key operations.

Guidelines for Setting Up a Micro-Production Unit and Establishing Inoculant Quality Control

1. Physical Facility

A conceptual plan for a MPU facility addresses the environmental requirements of specific functions. (See Fig. 1. MPU: Suggested floor plan.) The plan may be modified as needed. This layout includes six functional sections:

A) office area,
B) quality control testing area,
C) general area for media prep, glassware washing, etc.,
D) inoculant curing and short-term storage,
E) broth injection area, and
F) clean area for microbiological work.

This plan is based on the assumption that only pre-packaged sterile peat carrier will be used for production. No provisions are made for processing locally-acquired peat. If indigenous peat will be used, then drying, grinding, weighing, and packaging must be done in another area away from the MPU. Dust control is necessary in the peat processing area.

An isolated area must be designated for the microbiological work. (See Fig. 2. MPU culture and injection area.) Non-essential personnel and materials must be kept out, and traffic should be restricted. Double entrance doors reduce dust. Air supply (air conditioners) to the clean room needs to be effectively filtered to avoid the introduction of contaminants. Maintenance of positive air pressure in the room helps reduce the introduction of unfiltered air from outside. Soil, raw peat, and plant tissue samples must not be brought into this area without special precautions for containment, handling, and disposal.

2. Equipment and Supplies

Table 5 includes general equipment items useful in the production area. Items specific to microbiological activities are listed in Table 6. (Microbiological equipment and supplies checklist.)

3. Strain Transfer, Cell Propagation, and Broth Dilution

A) Preparation of Inoculants

Small-scale inoculant production with the MPU takes advantage of inexpensive fermentors and simple techniques. (See Fig. 3. MPU production flow.)

A starter culture is introduced aseptically to a fermentor such as a modified Erlenmeyer flask. (See Fig. 4. MPU: Steps for inoculation of fermentor.) Rhizobial growth proceeds under standard conditions: **28-30°C** for 3-5 days for fast growers and 5-7 days for slow growers. The culture may be aerated by rotating on a orbital shaker or by bubbling air in through a sterile filter.

B) Dilution of Broth and Injection into Carrier

After maturation, the broth is diluted with water or 1 /4 strength Yeast Mannitol Broth (YMB) (See Table 7 for ingredients) at a ratio of 1:100. (See Fig. 5. Dilution and manual injection of broth into sterile peat carrier, and Fig. 6. Self-refilling syringe for injecting diluted broth into carrier.)

For small-scale production, the use of a manual hand-syringe and separate sterilizable dilution container is recommended. For medium to commercial-scale production, either a peristaltic pump system or the dilution-dispensing pump system are more efficient. (See Fig. 7. Dilution and dispensing of rhizobial broth by pump into peat carrier, and Fig. 8. Diluting and dispensing pump system.) Diluted broth is injected into a sterile, pre-packaged carrier material such as peat. The package is then massaged to mix the diluted broth and carrier, and left for 5-7 days at 28-30°C. (See Table 4. Time required for injecting peat bags.)

4. Rhizobial Strains

Pure cultures of authenticated rhizobia must be obtained from internationally recognized sources and must be specifically recommended for target legumes. Strains must have been tested for purity and effectiveness on their host plants.

Important characteristics of rhizobia include

A) ability to compete with indigenous rhizobia in the soils
B) ability to adapt to the environmental conditions encountered in the soil
C) ability to persist in the soil
D) ability to fix nitrogen effectively with local legume genotypes and cultivars.

One of the advantages of a small-scale MPU system is that authenticated, selected, and pre-tested rhizobial cultures may be obtained in a preserved form. These cultures can be reconstituted as a liquid culture for direct transfer to a fermentor (see Fig. 4.) Multiple samples of preserved inoculant strains may be obtained and stored for later use. This method reduces the likelihood of contamination, and eliminates the need for maintaining a rhizobial culture collection and authenticating cultures on plants.

5. Carrier Material

The following analysis of an Australian sedge peat marketed by Bio-Care Technology, New South Wales can be used as reference when characterizing other potential carrier materials.

The initial pH ranges between 4 and 5, depending on the depth from which it is mined. The pH must be adjusted to 6.5 - 7.2 by adding CaC03 at a rate of approximately 5% (w/w).

The initial moisture content is approximately 15%. Peat sterilized by gamma irradiation, steam sterilization, or dry heat and may vary in moisture content, which affects the amount of diluted broth which can be mixed with the carrier material.

Particle size after grinding varies by batch. More than 90% of the final peat carrier will pass through 325 mesh (50 ,um) screen.

The Bio-Care peat carrier material is sterilized by Cobalt 60 irradiation and has the following characteristics.

Organic matter:	80-85%
Cation exchange capacity :	80 mEq/100 g
Water holding capacity:	200 % (w/v)
Chemical composition:	

N %	1.90 - 2.40
P %	0.03
K %	0.06
Mg%	0.57 - 0.64
Ca %	1.10 - 1.60
Na%	0.22
Cl%	0.10 - 0.11
Al %	1.40 - 1.70
Fe %	0.20 - 0.40
Cu	5 - 30 ppm
Zn	15 - 65 ppm
B	4 - 10 ppm
NaCl	0 - 0.15

6. Labels for Rhizobial Inoculants

Requirements for labelling inoculants vary among countries. (See Fig. 9. Sample label for inoculants.) Each packet of inoculant should contain the following information:

A) name, address, phone and FAX numbers of the inoculant production facility
B) name of legume host
C) expiration date after which the inoculant should not be used
D) batch number
E) precautions
F) instructions for storage
G) directions for use
H) net weight of unit size produced
I) quantity of seed to be inoculated

7. Quality Control

A) Industry Standards

Inoculant quality is ultimately judged by the farmer in the field. Once produced, the inoculant is subject to a long chain of "unknown factors" that may affect its quality. Temperature, desiccation, poor aeration, pH drift, contamination, and toxicity from the carrier material may all cause deterioration of the product. One of the advantages of MPU-produced inoculants is that they retain their quality and shelf-life longer than inoculants produced in non-sterile carriers.

Minimum standards of acceptability are based on the number of viable rhizobia per gram of inoculant. These standards vary between countries. For example:

5-10 x 10^7 viable cells/gram - USSR
4-25 x 10^9 cells/gram - The Netherlands
1 x 10^8 cells/gram - Australia, New Zealand, India, and Czechoslovakia

B) Plant Growth Area for Testing Inoculants

The primary advantage in quality control procedures when using a sterile carrier is that the rhizobia in the inoculant can be enumerated directly by plating methods. When these direct plating methods are combined with other presumptive tests for rhizobia, indirect enumeration of rhizobia by Most-Probable-Number (MPN) plant infection methods are not required.

Depending on the situation, environmentally-controlled areas for plant growth can be used to:

1) authenticate cultures received from germplasm depositories,
2) estimate the numbers of infective rhizobia in soils or non-sterile inoculant carriers using Most Probable Number (MPN) plant infection tests, and
3) measure the effectiveness of strains on the plant.

Primary considerations for the physical design of the plant growth room are:

1) protection from outside contamination by other rhizobia and microorganisms,
2) adequate lighting (approximately 16,000 lux),
3) adequate relative humidity (65 - 70%),
4) air circulation, and
5) ventilation of excess heat to maintain a temperature of approximately 20°C during light period and 15°C for dark period.

(See Fig. 1. MPU: Suggested floor plan, and Fig. 10. Plant growth area.)

If the area is exposed to adequate sunlight, greenhouse-type construction should be considered. Temperature and humidity must be regulated to support plant growth.

If the area is not exposed to sufficient natural light, an enclosed growth-room with artificial sources of illumination should be considered. Warm-white or cook white fluorescent lighting mounted to the wall or suspended over-head are often used. Wall-mounted lighting does not encourage excessive vertical growth in plants, and is often preferred. The ballasts for fluorescent lights creates heat and should be located outside the growth area. An alternate lighting system is a high-intensity discharge sodium lamp, but this system generates high temperatures and is costly. Day-length with the artificial lights must be regulated by timers. Depending on the legume species, lighting periods are generally 12 to 16 hours.

C) Quality Control Measures

Broth cultures should be tested by presumptive tests such as gram stains, direct microscopy, plating on indicator media, measuring pH, etc. Rhizobia in inoculants can be enumerated by direct-plating techniques when inoculants are produced with sterile carriers. This direct plate count will also reveal the presence of any contaminating organisms.

It must be emphasized that the direct-plating method is only applicable to inoculants produced by sterile technique. Most contaminating organisms grow faster than rhizobia and will interfere with the direct-plate count. Any contamination indicates the need to further test the inoculant batch.

The plant infection technique provides indirect estimates of rhizobial populations based on Most-Probable-Number (MPN) tables or the MPNES computer-assisted program. MPN assumes that nodulation will occur if a single viable and infective rhizobia is present, and that the lack of nodules indicates the absence of compatible, viable rhizobia.

The only way to ensure that the symbiotically correct rhizobial strain for a specific legume host is in the inoculant is to test the inoculant on the host. It is conceivable that inoculants may have adequate populations of rhizobia, but through some error, these rhizobia may not be the correct strain for the intended host.

D) Testing Inoculants by Batch Lot Numbers

The Australian Inoculants Research and Control Service (AIRCS) test commercially-prepared inoculants by the direct plate counting technique. AIRCS recommends random selection of seven units from a batch.

- five of these units are tested initially
- if one unit fails on initial testing, it is re-tested
  - if, on the second trial it meets all criteria, the entire batch lot is passed
  - if, on the second trial it fails,
    - the additional two units are tested
      - if either of these units fails, the entire batch lot fails

Inoculant samples selected at random for testing should be kept at 2-10°C.

The sampled units should be selected on a Batch Lot Number basis. If a problem is detected in samples selected from a specific batch lot number, units of that specific batch could be further tested to determine if the problem was isolated to that batch or continued throughout production. Other batches that pass the quality control tests can be released for sales.

Producers should test inoculants from retail outlets to ensure that conditions associated with transportation and storage have not altered the quality of the inoculant. Samples from retail outlets should be stored in the refrigerator until they are tested.

8. Heating Requirements for Various Fermentor Volumes

NifTAL is testing the substitution of electric immersion heater probes for LPG (Liquid Propane Gas) burners for sterilizing fermentors. Some disadvantages of LPG burners include

A) use of an open flame,
B) excess heat,
C1 carbon and soot deposits,
D) location of the fermentor outside the laboratory away from heat, and
E) the need to move a full sterile fermentor back into a "clean" area for inoculation and fermentation.

Electric immersion heaters address each of the disadvantages and offer a superior alternative compared to LPG burner use. The greatest advantage of electric heating is the convenience of sterilizing the fermentor in the same area where inoculation and rhizobial production will take place, usually a clean laboratory. In contrast, the use of LPG generates too much heat for this situation. However, LPG use may be advantageous for other reasons in some situations.

By using an LPG gas burner rated at 90,000 BTU capacity, 100 litres of non-sterile broth in a stainless steel 141 litre NifTAL-developed fermentor can be sterilized in approximately 90 minutes. The broth is heated from (70°C) to (251 °C), held at 251 °C for 30-45 minutes, then cooled by internal coils circulating cold water. (See Table 8. Kilowatt hours required to heat water for different volumes and time, and Fig. 12 Kilowatt hours required to heat water.)

Tables and Figures

Table 1. Comparison of costs and operations time for sterile and non-sterile production mode.

Area To Be Planted (ha): 33,000

Production Mode : Non–Sterile

Fermentor vessel working capacity (litres) :	1	2	4	12	30	50	100
No. Units required :	680	340	170	57	21	14	7
fermentor cycles:	10	10	10	10	10	9	9
days of operation:	87	87	87	87	94	85	85
Fermentor(s) Cost:	$37,400	$28,900	$14,450	$126,390	$53,705	$36,638	$53,144

Production Mode : Sterile

Dilution rate = 100 : 1

Fermntor working capacity (liters):	1	2	4	12	30	50	100
No. Units:	1	1	1	1	1	1	2
Fermentor(s) Cost:	$374	$289	$145	$2,217	$2,557	$2,617	$7,592

Table 2. Fermentor capacities and operations time to meet production goals.

Dilution Rate to be used :	100	: 1
Seed Planting Rate :	50	kg/ha
Inoculant Application rate:	10	g/kg seed

Area to be Inoculated (ha)	Total Peat required (kgs)	Full–Strength Broth required (l)	Operations Time	Fermentor working capacity 1 litres	2 litres	4 litres	12 litres	30 litres	50 litres	100 litres
				1 units	1 units	1 units	1 units	1 units	1 units	2 units
1,000,000	300,000	2,000	Fermentation cycles*	2000	1000	500	167	67	40	10
			Total days required**	18000	9000	4500	1500	600	360	90
500,000	150,000	1,000	Fermentation cycles	1000	500	250	83	33	20	5
			Total days required	9000	4500	2250	750	300	180	45
100,000	30,000	200	Fermentation cycles	200	100	50	17	7	4	1
			Total days required	1800	900	450	150	60	36	9
33,000	9,900	66	Fermentation cycles	66	33	17	6	2	1	0
			Total days required	594	297	149	50	20	12	0
10,000	3,000	20	Fermentation cycles	20	10	5	2	1	0	0
			Total days required	180	90	45	15	6	3.6	0.9
5,000	1,500	10	Fermentation cycles	10	5	3	1	0	0	0
			Total days required	90	45	23	8	3	1.8	0.5
1,000	300	2	Fermentation cycles	2	1	1	0	0	0	0
			Total days required	18	9	5	1.5	0.6	0.4	0.1

* 9 days required to prepare a fermentor, inoculate, propagate, harvest, and then prepare the fermentor for inoculation again.
** A Fermentation cycle assumes that a period of 9 days are required for related activities. (# Cycles x 9 = Total days required)

Assumptions Made for Production Scale Model:

A. Broth requirements are based on final inoculant moisture content of 40%
B. Full strength broth x broth dilution factor = amount diluted broth required
C. Fermentor vessels 12, 30, 50, and 100 liter sizes are stainless steel pressure vessels
D. Fermentor vessels less than 12 liters are Erlenmeyer flasks.

Table 3. Potential production capacities of sterile and non-sterile production modes.

Assumptions:

Unit size (grams) :	250	
Dilution used for "Sterile" method:	100	
Cultured broth required/250 g unit	100	ml
Peat required/unit :	150	g
Inoculant required/kg seed:	5	g
Seed planting rate :	75	kg/ha

Operation Modes ------>		Potential production of 250 gram units	
		Non-Sterile	Sterile
Fermentor working volume (liters):	1	10	1,000
	12	120	12,000
	30	300	30,000
	100	1,000	100,000

		Potential hectares inoculated per fermentor volume	
Fermentor working volume (liters):	1	7	667
	12	80	8,000
	30	200	20,000
	100	667	66,667

Table 4. Time requirements for injecting peat bags.

Broth Injection Rate volume (ml)	time (min)	Handling Time/Unit (a) time (secs)	Total Unit Weight (finished) (b) (grams)
200	1	15	250

Number of 250 g bags for 1000000 ha			Output Capacity No. of 250 g bags/hr	Production Days* required	Months
2,000,000	20	Station (c)	1,920	174	5.8
	40		3,840	87	2.9

* Days are based on 6 hours

Number of 250 g bags for 500000 ha			Capacity	Days*	
1,000,000	10	Stations	960	174	5.8
	20	Stations	1,920	87	2.9

Number of 250 g bags for 100000 ha			Capacity	Days*	
200,000	2	Stations	192	174	5.8
	4	Stations	384	87	2.9

Number of 250 g bags for 33000 ha			Capacity	Days*	
66,000	1	Station	96	115	3.8
	2	Stations	192	57	1.9

Number of 250 g bags for 10000 ha			Capacity	Days*	
20,000	1	Station	96	35	1.2

Number of 250 g bags for 5000 ha			Capacity	Days*	
10,000	1	Station	96	17	0.6

Number of 250 g bags for 1000 ha			Capacity	Days*	
2,000	1	Station	96	3	0.1

(a) : Handling time/unit accounts for operator time to retrieve, puncture, inject, apply adhesive patch, and transfer unit to assistant for massage

(b) : Finished unit weight is peat + broth

(c) : Station is defined as one operator injecting broth into peat

Table 5. General equipment items.

Item Description	Type
Refrigerator	upright
Electrical generator	gas
Lab gas, portable bottle or piped	LPG/butane
Hot water heater	
Water treatment - (reverse osmosis),(soft water), (de-mineralization)	
Water pre-treatment - coarse filtration, activated charcoal	
Dehumidifier or desiccant for microscope	
Deep well sinks, corrosion resistant	stainless steel or PVC
Sink traps and waste pipes, corrosion resistant	PVC
Counter tops, chemical resistant	Plastic
Air conditioning, inoculant curing/ storage area, office, clean room	
Water hose outlets for clean-up	injection area
Floor drains to outside sump	injection area
Fluorescent lamps lighting	low output

Table 6. Microbiological equipment and supplies checklist.

Quantity	Equipment and Supplies	Size/type
	Microscope	light
	Steam sterilizer/ autoclave	electric
	Incubator, microbiological	convection
	Transfer chamber	
	Ultraviolet sterilizer for fluids	
	Ultraviolet lamps, spare	
	Surge suppressor	
	Balance, sensitivity 0.1 g	top load
	Shaker table	orbital
	Mixer, vortex type	
	pH meter	
	Flask, Erlenmeyer, screw cap	250 ml
	Flask, Erlenmeyer	1000 ml
	Flask, Erlenmeyer	2000 ml
	Flask, Erlenmeyer	4000 ml
	Stoppers, rubber, two hole	# 9
	Stoppers, rubber, two hole	# 10
	Bottle, dilution, screw cap	200 ml
	Tubing, glass, 10 meters	7.0 mm (o.d.)
	Syringes, glass, reusable	10 ml
	Syringes, glass, reusable	50 ml
	Hose clamps	Hoffman type
	Tubes, culture, screw cap	16 x 125 mm
	Rack, test tube	16 mm holes
	Scalpel blades	#10
	Forceps	dressing type

Quantity	Equipment and Supplies	Size/type
	Petri dishes, glass	100 x 15 mm
	Needles, hypodermic, reusable	12 gauge
	Needles, hypodermic,	18 gauge
	Canister, pipette, Pasteur pipet	
	Pipettes, Pasteur	23 cm length
	Inoculating loop	
	Tape, indicator sterilization	
	Weighing paper	15 x 15 cm
	Cotton, non-absorbent	2 lbs.
	Aluminum foil	25 meters
	pH meter buffer solution	pH 4.01
	pH meter buffer solution	pH 7.0
	pH meter buffer solution	pH 10.0
	pH electrode storage solution	500 ml
	pH meter filling solution	500 ml
	Latex tubing, 15 meters	6.4 x 1.6 mm
	Latex tubing, 15 meters	4.8 x 0.8 mm
	Gram stain set	250 ml
	Microscope slides	
	Immersion oil	
	Cover slips	24 x 50 mm
	Tape, label	

Laboratory name:

Location:

PRELIMINARY ESTIMATES

Facility Production capacity: 10 metric tonnes

Date prepared: Jul-95

Start-up : Fixed captital cost equipment and supplies

No.	Item description	Type	Source	Cat. No.	Unit	Qty. /Unit	Estimated US$	Comments
1	Aspirator	Faucet, vacuum producing	Fisher	09-956	each	1	$49.00	
2	Autoclave, steam, bench-top	chamber: 26L x 16" dia.-240V	Fisher	14-460-10	each	1	$7,665.00	
3	Balance, hanging pan, dial	0.01 g sensitivity-410g capacity	Fisher	02-020-410	each	1	$257.60	
4	Bars, magnetic stirring	Set - assorted sizes	Fisher	14-511-96	set	5	$50.40	
5	Bars, magnetic stirring	Egg-shaped - 3/4" dia. x 2 1/2" l	Fisher	14-511-58C	each	1	$70.00	
6	Beakers	150 ml - Fleaker style	Fisher	02-599-15	pack	10	$50.60	
7	Beakers	500 ml - Fleaker style	Fisher	02-599-19	pack	10	$64.43	
8	Beakers	1000 ml - Fleaker style	Fisher	02-599-23	pack	10	$105.38	
9	Beakers, caps	150- 500 ml for Fleaker style	Fisher	02-599-27	pack	10	$83.17	
10	Beakers, caps	800- 1200 ml for Fleaker style	Fisher	02-599-28	pack	10	$121.76	
11	Beaker, gripper	for Fleaker type beakers	Fisher	02-599-29	each	1	$5.21	
12	Bottle, dilution	screw cap, 200 ml	Fisher	02-945-10	case	48	$120.29	
13	Burner	lab type - LP gas	Fisher	03-962P	each	1	$44.80	
14	Canister, pipette stainless steel	For Pasteur pipettes, 5x5x23 cm	Fisher	03-475-5	each	1	$40.60	
15	Carboy, w/o spigot	clear polycarb.-9 l cap.-100 mm cap	Fisher	02-961-55A	each	1	$87.99	
16	Carboy, w/spigot	clear polycarb.-10 l cap.-83 mm cap	Fisher	02-962-7G	each	1	$68.65	
17	Carboy, spigot replacement	Tefzel ETFE - autoclavable spigot	Fisher	02-963-10A	each	1	$123.20	
18	Carboy, vent	Filling/Venting closure	Fisher	02-923-15	each	1	$21.00	
19	Connectors, tubing	Y-shaped, glass, 1/4" od	Fisher	15-333A	pack	12	$44.10	
20	Connectors, tubing	Y-shaped, 5/16" id	Fisher	15-315-30C	pack	12	$14.62	
21	Connectors, tubing	Y-shaped, 3/8" id	Fisher	15-315-30D	pack	12	$20.66	
22	Connectors, tubing	Reducing connector, 3/8 to 1/4"	Fisher	15-315-6B	pack	12	$14.70	
23	Connectors, tubing	Reducing connector, 1/2 to 3/8"	Fisher	15-315-6C	pack	12	$14.70	
24	Cylinders, graduated	25 ml - clear polymethylpentene	Cole-Palmer	G-06134-25	each	1	$5.67	
25	Cylinders, graduated	100 ml - clear polymethylpentene	Cole-Palmer	G-06134-45	each	1	$8.54	
26	Cylinders, graduated	500 ml - clear polymethylpentene	Cole-Palmer	G-06134-65	each	1	$15.89	
27	Cylinders, graduated	1000 ml - clear polymethylpentene	Cole-Palmer	G-06134-75	each	1	$19.81	
28	Dishes, Petri, bacteriological	glass, reusable, 100x15 mm	Fisher	08-746C	case	72	$267.12	
29	Fermentor vessel	20L working capacity - electric steriliz	BNF Industries-1995	BNF-20L	each	1	$6,174.00	
30	Fermentor vessel, accessories	aeration system for all models	BNF Industries-1995	BNF-Air	each	1	$610.40	
31	Fermentor vessel, accessories	spare parts kit for all models	BNF Industries-1995	BNF-SP kit	each	1	$742.00	
32	Flask, Erlenmeyer	1000 ml - tubulation	Fisher	10-180L	case	6	$225.29	
33	Flask, Erlenmeyer	screw cap, 125 ml	Fisher	10-093A	pack	6	$105.81	
34	Flask, Erlenmeyer	screw cap, 250 ml	Fisher	10-093B	pack	6	$109.63	
35	Flask, Erlenmeyer	screw cap, 500 ml	Fisher	10-093C	pack	6	$112.59	
36	Flask, Erlenmeyer	2000 ml - tubulation	Fisher	10-180M	each	1	$92.22	
37	Flask, Fernbach	2800 ml	Fisher	10-092	each	1	$43.46	
38	Forceps	tissue	Fisher	13-812-36	each	1	$14.00	
39	Funnels, plastic	72 x 7 mm (top/stem)	Cole-Palmer	G-06120-30	pack	12	$24.78	
40	Funnels, plastic	166 x 14 mm (top/stem)	Cole-Palmer	G-06120-70	pack	2	$20.51	
41	Hood, laminar flow, HEPA filter	360 cfm, 60x60x60 cm work area	Fisher	92-950	each	1	$1,999.00	
42	Hose clamps	Hoffman open side: 5/8-1"	Fisher	05-875B	pack	10	$28.00	
43	Hose clamps	Hoffman open side: 1/2-3/4"	Fisher	05-875A	pack	10	$28.00	
44	Incubator, microbiological	3.1 cu.ft capacity-230V 1.5A	Fisher	11-683-631D	each	1	$1,190.00	
45	Inoculating loop	Bacteriological - 76mm 25g wire	Fisher	13-066	case	12	$53.20	
46	Microscope, binocular	10x-40x-100x(oil)	Fisher	12-576-2D	each	1	$1,335.60	
47	Mixer, test tube	Vortex type - 240V 50/60Hz	Fisher	12814-4	each	1	$211.40	
48	Needles, hypodermic-s/steel	16 gauge, steel	Fisher	14-825M	pack	12	$95.20	
49	Needles, hypodermic-s/steel	13 gauge, steel	Fisher	14-826-33	pack	12	$156.80	
50	Pail, plastic	5 gal. - polyethylene	Cole-Palmer	G-06274-25	each	1	$7.00	

Laboratory name:

Location:

PRELIMINARY ESTIMATES

Facility Production capacity: 10 metric tonnes

Date prepared
Jul-95

No.	Item description	Type	Source	Cat. No.	Unit	Qty. /Unit	Estimated US$	Comments
51	Pail, plastic lid	Resealable lid with gasket	Cole-Palmer	G-06274-35	each	1	$2.80	
52	pH meter	Benchtop meter	Cole-Palmer	G-05669-20	each	1	$385.00	
53	pH meter cable	Cable for electrode	Cole-Palmer	G-05669-52	ft.	3	$42.70	
54	pH meter electrode	Triaxial "3-in-1 style"	Cole-Palmer	G-05669-50	each	1	$96.60	
55	pH meter voltage adapter	Adapter, 230 VAC	Cole-Palmer	G-08403-55	each	1	$16.80	
56	Pipettes, Pasteur	controlled drop-15.2cm lgth.	Fisher	13-678-6C	case	1000	$79.80	
57	Pipettes, serological	1.0 ml - cotten plugging	Fisher	13-671-106B	case	12	$69.38	
58	Pipettes, serological	5.0 ml - cotten plugging	Fisher	13-671-106E	case	12	$73.42	
59	Pipettes, serological	10 ml - cotten plugging	Fisher	13-671-105H	case	12	$84.00	
60	Pump, aeration	aquarium type			each	1	$140.00	
61	Pump, diluter/dispenser	Variable volume/dilution ratio, 240VAC	Oyster Bay Pump	Single Channel	each	1	$4,650.00	
62	Pump, diluter/dispenser	Additional dispensing channels	Oyster Bay Pump	Multi-Channel	each	1	$1,200.00	
63	Pump, hand syringe	Spare glass barrel	Wheaton	851397	pack	1	$19.66	
64	Pump, hand syringe	Spare valve assembly	Wheaton	851431	pack	1	$69.55	
65	Pump, hand syringe	4-20 ml capacity-self-refilling	Wheaton	851377	pack	1	$229.60	
66	Rack, test tube	16 mm holes	Fisher	14-796-1	each	1	$23.80	
67	Scapel blades	No. 10	Fisher	08-916-5A	pack	100	$97.30	
68	Scapel handle	stainless steel	Fisher	08-913-5	each	1	$15.40	
69	Scissors	Operating, blunt/sharp, 5 1/2" l	Fisher	13-808-2	each	1	$21.84	
70	Scissors	Utility cutter	Fisher	14-277	case	6	$82.60	
71	Scoop, plastic	Polycarbonate, 6 oz.	Cole-Palmer	G-66600-00	each	1	$5.95	
72	Scoop, plastic	Polycarbonate, 32 oz.	Cole-Palmer	G-66600-20	each	1	$10.85	
73	Scoop, plastic	Polystyrene, 2 oz.	Cole-Palmer	G-06260-30	pack	108	$69.90	
74	Sealer, thermal, plastic bag	24" sealing length, floor-110 VAC	Consolidated Plastics	92972KC	each	1	$665.00	
75	Sealer, spare repair kit	teflon-element-adhesive strip	Consolidated Plastics	92973KC	each	1	$82.39	
76	Shaker table for broth cell cultures	platform-universal (13x22")	Fisher	14-278-13	each	1	$1,316.00	
77	Shaker table for broth cell cultures	Orbital - table top	Fisher	14-278-5	each	1	$518.00	
78	Stirrer, magnetic, lab use	5 1/2"dia.x3 1/4"H-240V 50Hz	Cole Palmer	G-84000-05	each	1	$280.00	
79	Stirrer, magnetic, fermentor tanks	4 3/4"dia.x2 3/4"H-240V 50Hz	Fisher	14-493-121S	each	1	$194.60	
80	Stoppers, rubber	No. 13	Fisher	14-140Q	pack	4	$21.00	
81	Stoppers, rubber	No. 10	Fisher	14-140M	pack	8	$21.00	
82	Stoppers, rubber	No. 9	Fisher	14-140L	pack	11	$21.00	
83	Stoppers, rubber	No. 8	Fisher	14-140K	pack	13	$21.00	
84	Syringes, glass	50 ml	Fisher	14-826-14	case	120	$154.56	
85	Syringes, glass	30 ml	Fisher	14-823-2C	case	120	$100.02	
86	Syringes, glass	10 ml	Fisher	14-826-13	pack	100	$29.23	
87	Tubes, culture	screw cap, 16x125 mm	Fisher	15-327B	case	144	$237.89	
88	Tubing, glass	5-4' lengths; 7 mm od x 1.1 id	Fisher	11-350E	ft/pack	20	$38.50	
89	Water treatment - Ultra violet sterilzer	8 litres/min. cap.-220V 50-60Hz	Imperial Sales, Inc.	SP-2	each	1	$470.00	
90	Water treatment - Ultra violet sterilzer	Spare UV lamp	Imperial Sales, Inc.	SP-2 lamp	each	1	$56.00	
91	Water treatment-DeMineralization	3-module Type 1 Kit, complete	Fisher	09-050-237	each	1	$518.00	
92	Water treatment-Reverse Osmosis	15-40 litres/hr: ROpure LP system	Fisher	09-034-125	each	1	$1,605.00	
93	Water treatment-Reverse Osmosis	Membrane-high flow Cellulose acetate	Fisher	09-034-177C	each	2	$603.00	
94	Water treatment-Soft Water treatment	Condition feed water to reverse osmosi	Fisher	09-034-55	each	1	$813.00	
95							$0.00	
96							$0.00	
97							$0.00	
98								
99								
100								

Table 7. Culture media used for inoculant production, Yeast Mannitol Broth (YMB)

Ingredient	Formula	Formula weight	grams/l	Final mM
Potassium phosphate, dibasic	K_2HPO_4	174.18	0.5	2.87
Magnesium sulfate, heptahydrate	$MgSO_4$-7 H_2O	246.48	0.2	0.81
Sodium chloride	NaCl	58.44	0.1	1.71
D-Mannitol	$C_6H_{14}O_2$	182.17	1.0	5.49
Yeast extract			1.0	

Figure 1. The MPU suggested floor plan.

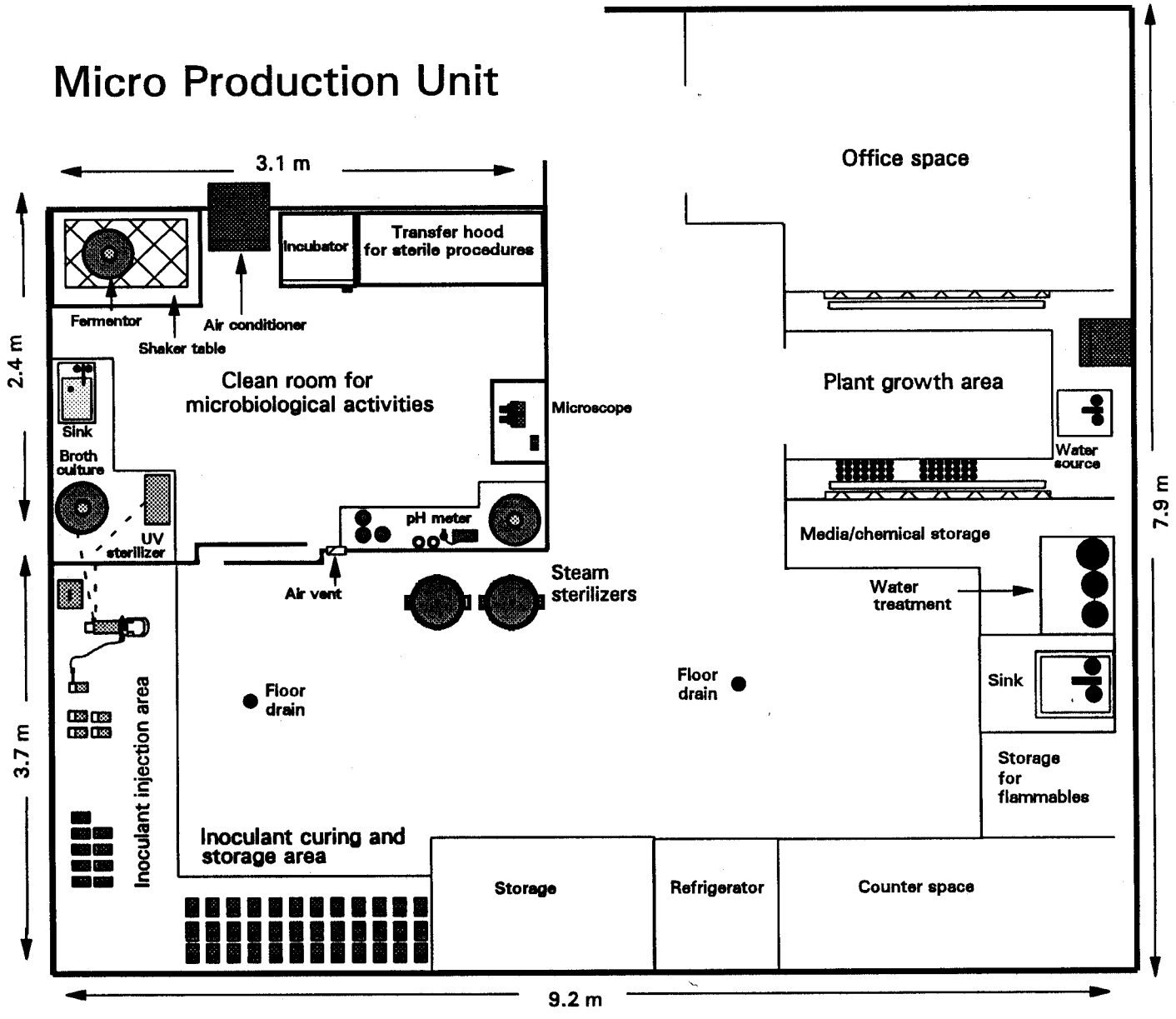


Figure 2. MPU culture and and injection area.

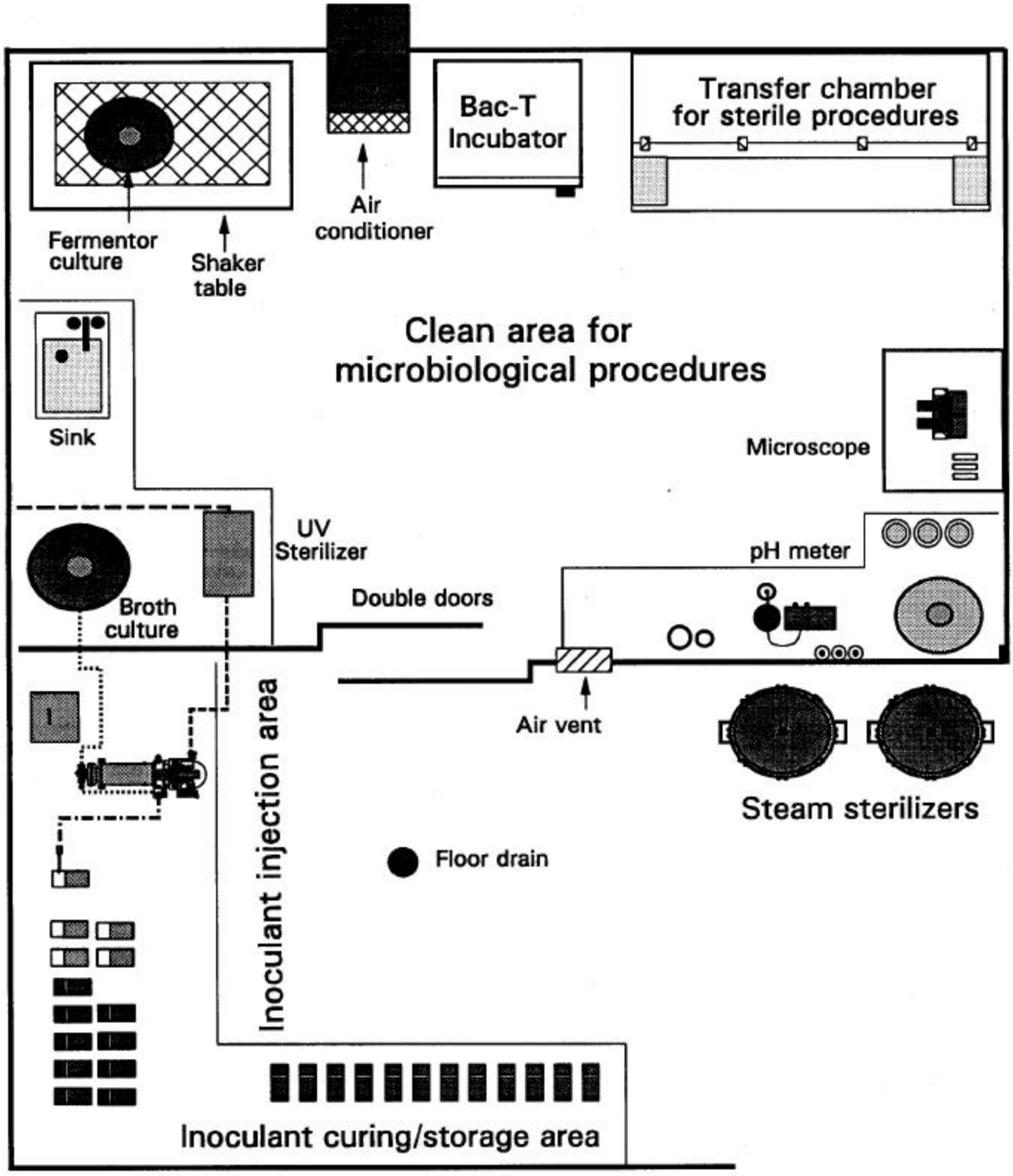


Figure 3. MPU production flow.

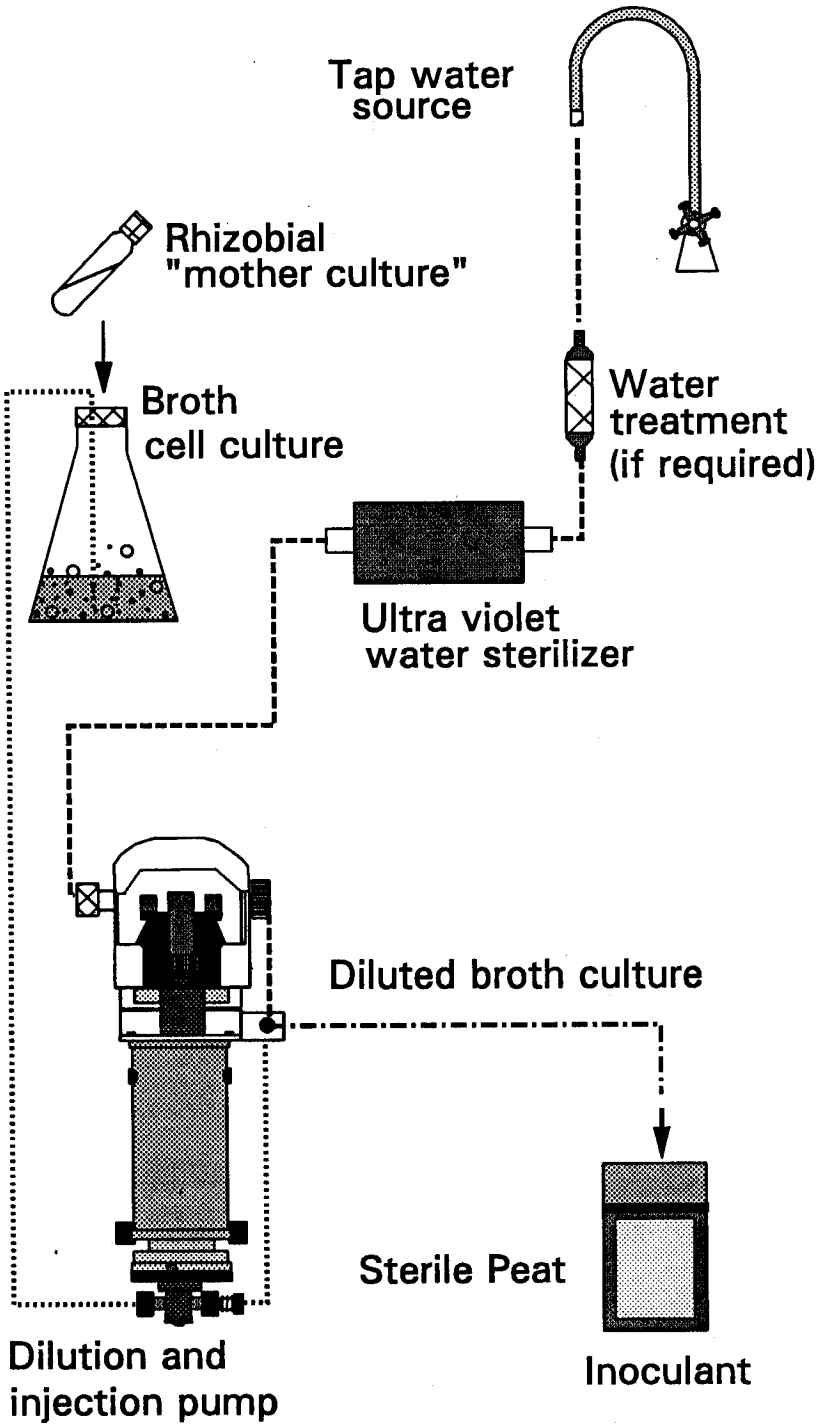
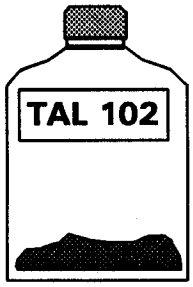
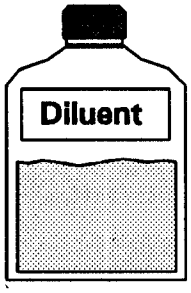


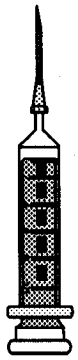
Figure 4. Steps for inoculation of fermentor.



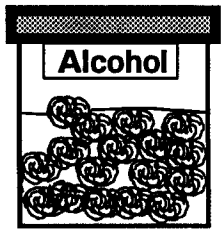
Preserved rhizobial culture



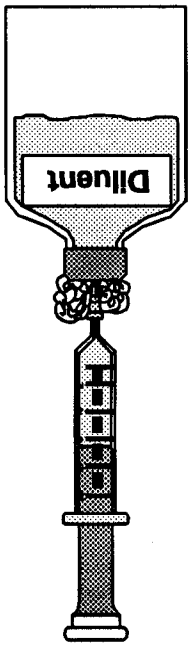
Sterile diluent

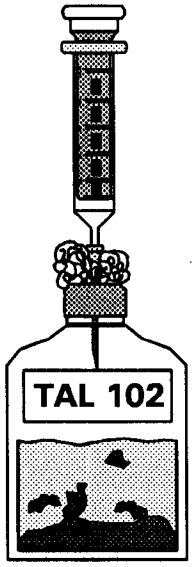


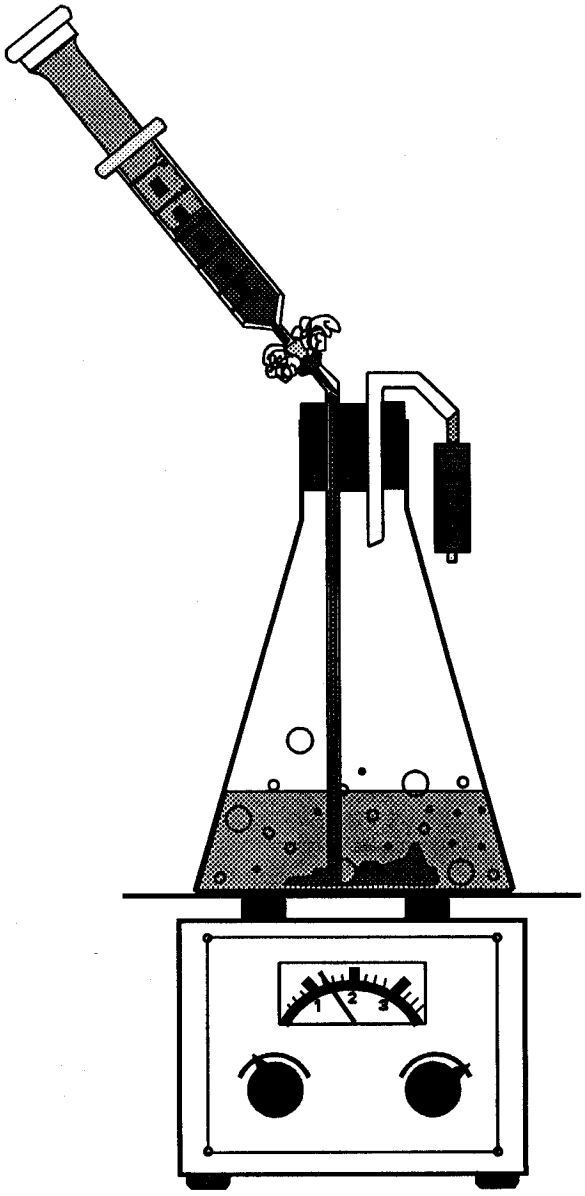
Sterile syringe



Cotton balls







1. Preserved culture is rehydrated with sterile diluent.

2. Culture is used to inoculate fermentor.

Figure 5. Dilution and manual injection of broth culture into sterile peat.

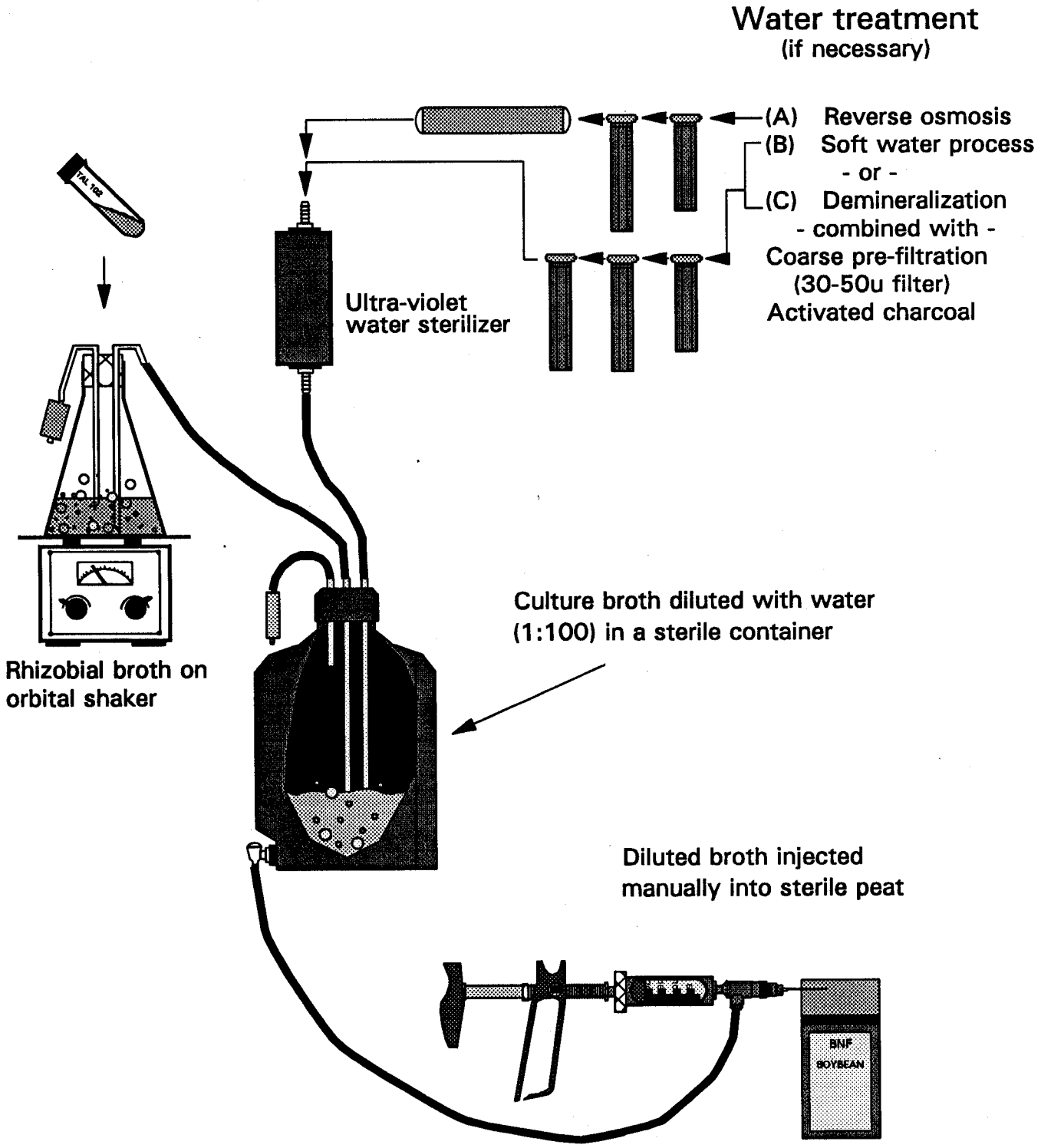


Figure 6. Self refilling syringe for injecting broth into carrier.

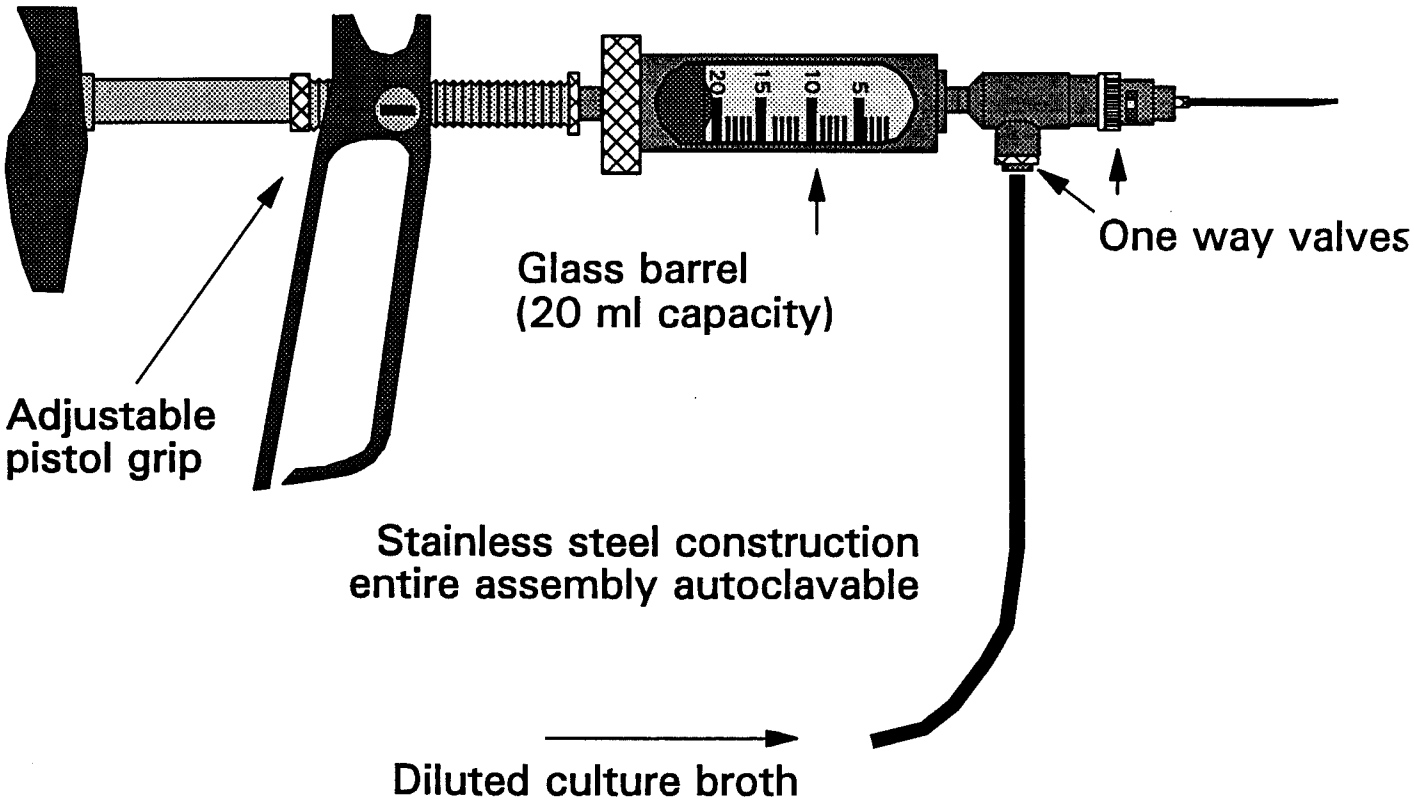


Figure 7. Dilution and dispensing of rhizobial broth by pump into peat carrier.

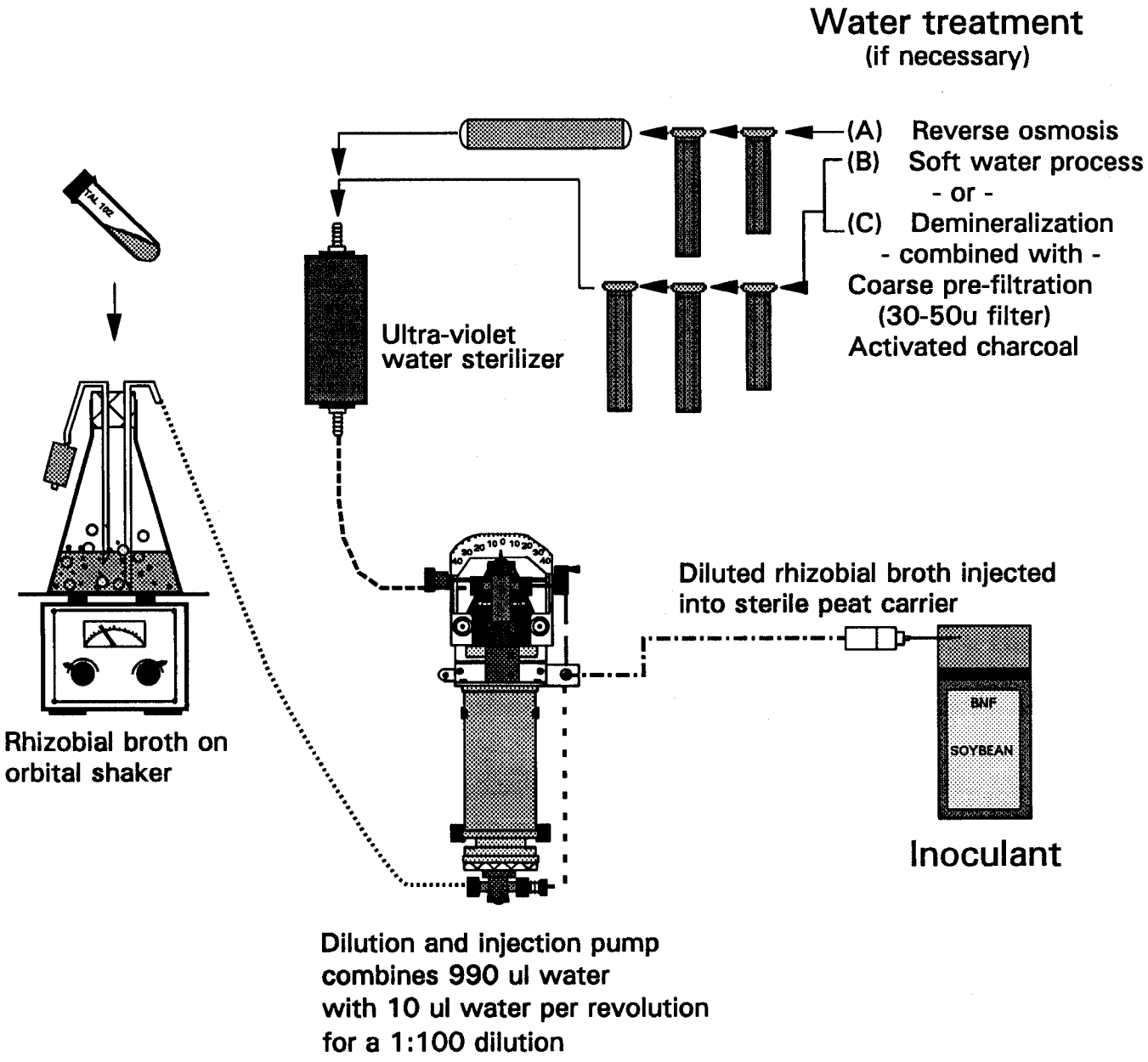


Figure 8. Diluting and dispensing pump system.

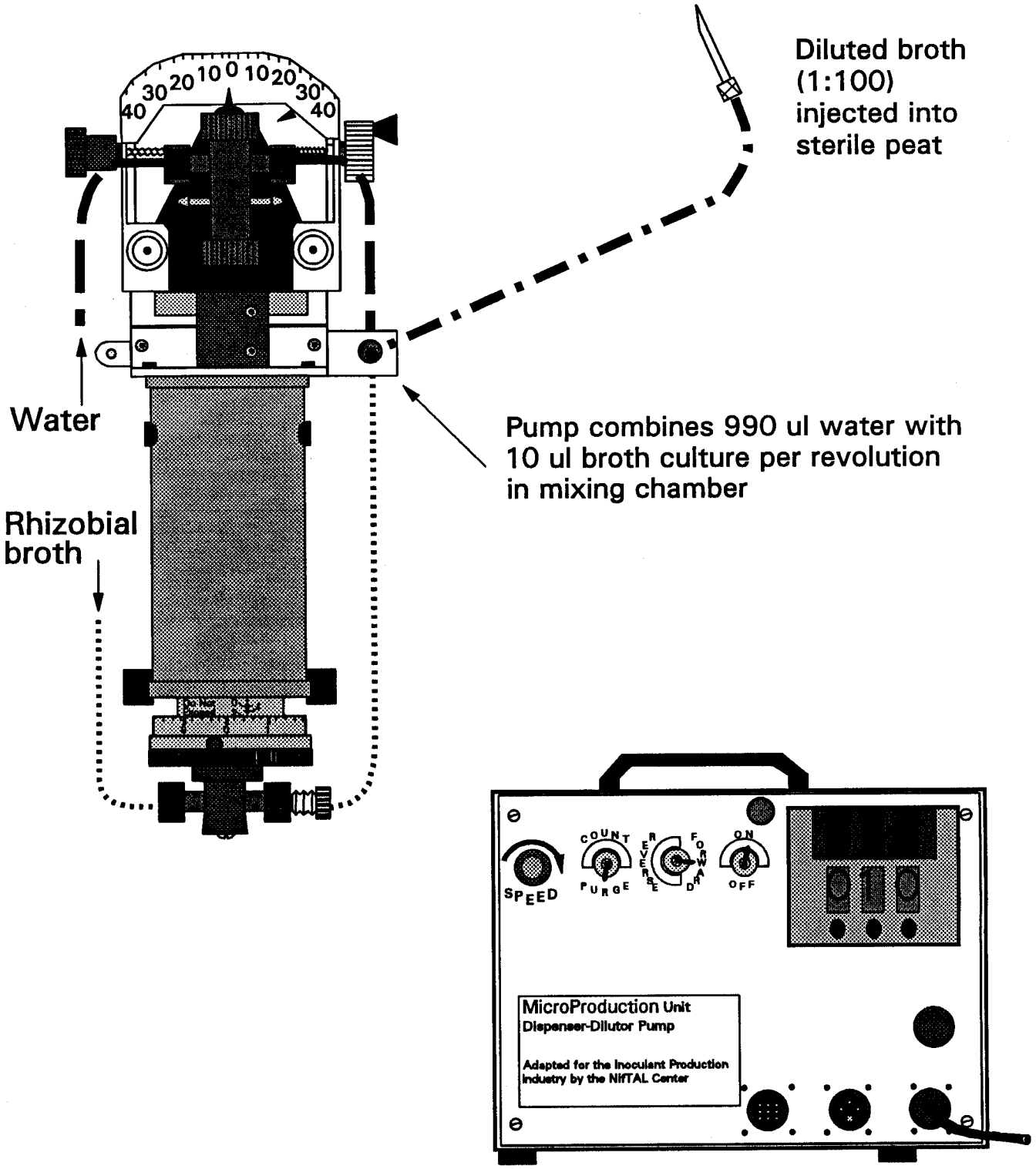


Figure 9. Sample label for inoculants.

BNF SEED COMPANY

BNF Seed Co. Inoculants, Inc.
P.O. Box 123
Anywhere, Country
011–2–61 234567
011–2–61 234789

LEGUME
INOCULANT FOR : **Soybean**

Batch Number : GM–17.3.93

Expiration Date: **December–1994**

Perishable: Inoculant contains LIVE nitrogen–fixing bacteria

DO NOT * Keep in direct sunlight
DO NOT * Use inoculant past expiration date
DO NOT * Store inoculated seed
DO NOT * Store at temperatures above 30 or below 4 degrees C
DO NOT * Freeze

INOCULATING SEEDS:

Sticker : To coat seed, use a commercial sticker
or use a sugar solution as described below.

1. Dissolve 1 part sugar to 9 parts water. Add 20 ml of sugar solution to 1 kilogram of seed. – OR – Add 20 ml of water to 1 kilogram seed if sugar is not available.
2. Add sticker to seeds and mix evenly in a plastic bag or bucket.
3. Add 10 g inoculant.
4. Mix inoculant and seed thoroughly in a cool, shady area, and air–dry about 30 minutes.
5. Protect inoculated seeds from direct sunlight, heat, and excessive drying.
6. Plant immediately.

Weight and Volume Guides :

A. 1 teaspoon (full) = 6 grams inoculum –OR– 6 ml water
B. 1 tablespoon (full) = 12 grams inoculum –OR– 10 ml water
C. 1 cup (loose pack) = 145 grams inoculum –OR– 250 ml water
D. 1 liter volume of Soybean seeds = 750 grams (about 6750 seeds)

Net Weight : 250 grams

Weight of seed this pack will inoculate : 25 (Kgs)

Figure 10. Plant growth area.

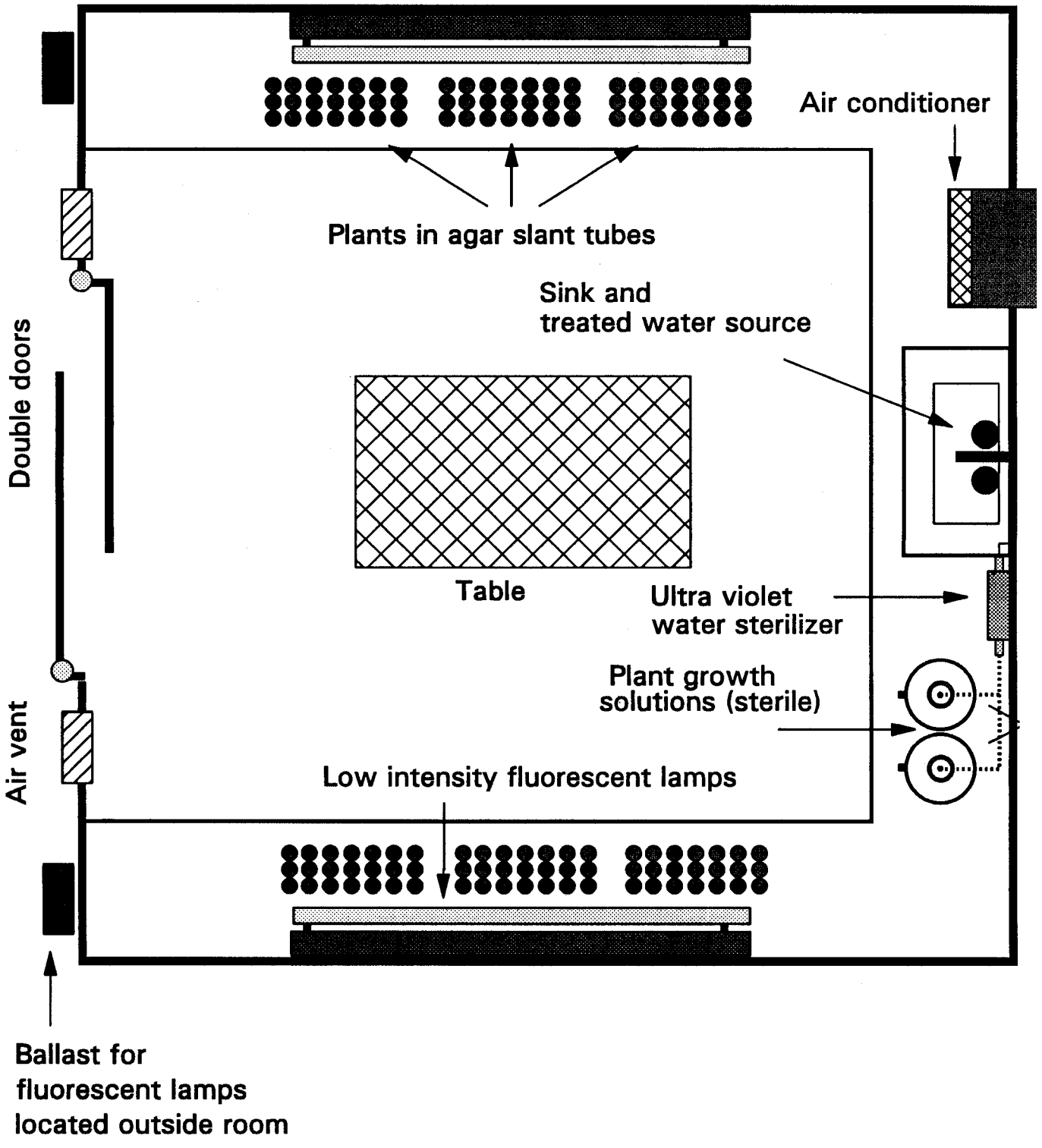


Figure 11. Quality control process.

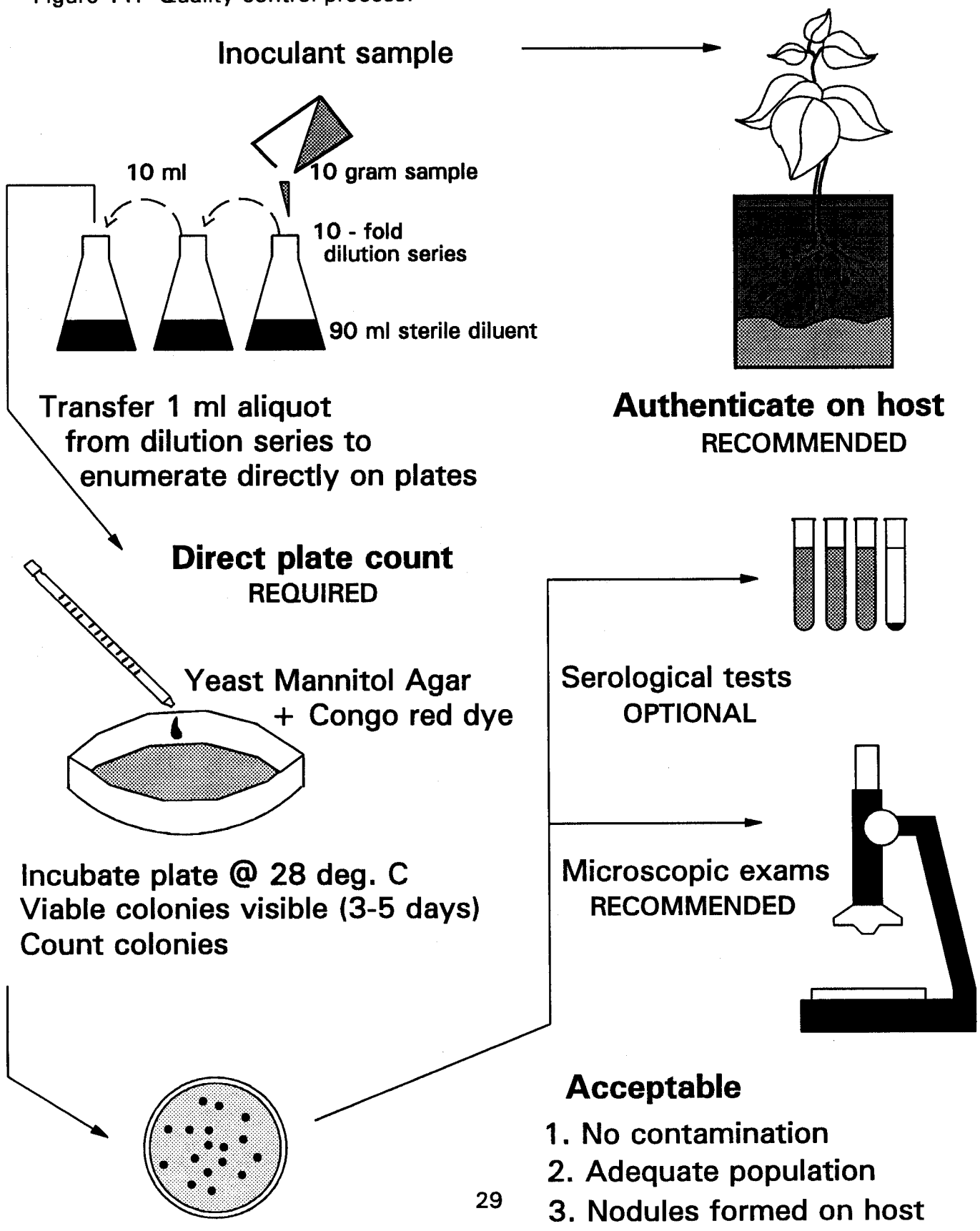


Table 8. Kilowatt hours to heat water for different volumes and times. See Figure 12.

Kilowatt hours required to heat water for different volumes

Working capacity (litres)	Temperature difference (deg F)	Heating time desired (hrs.)	Litres (12) Kilowatts required (kW)	(30) Kilowatts required (kW)	(50) Kilowatts required (kW)	(100) Kilowatts required (kW)	(300) Kilowatts required (kW)
12	100	0.25	3.90	9.76	16.26	32.52	97.55
30		0.5	1.95	4.88	8.13	16.26	48.78
50		0.75	1.30	3.25	5.42	10.84	32.52
100		1	0.98	2.44	4.06	8.13	24.39
300		1.5	0.65	1.63	2.71	5.42	16.26

Initial heating kW requirements

$$kWh = \frac{Qw+Qc}{3412} + \frac{LsH}{1000*f}$$

Sp. heat of steel: 0.12 BTU/lb–oF
Sp. heat of water: 1 BTU/lb–oF

Tank capacity (litres): 50
Heating time (hrs.) : 1.00

initial wt. of H20 in tank: 110.20 lbs.
Exposed surface area: 8.84 Ft 2
Temperature difference: 100 oF

Heat loss (from graph): 180.00 watts/ft 2 ----> Use : 10 (for insulated s/s vessel wall)
180 (for uninsulated s/s vessel wall)

BTU to kW conversion: 3412

watts to kW conversion: 1000

avg. factor over time: 2

Qw = 11,020 BTU
Qc = 540 BTU
Ls = 1,591 watts
H = 1 hrs.
f = 2

Initial heating capacity in kW : 5.0

Maintenance kW requirements

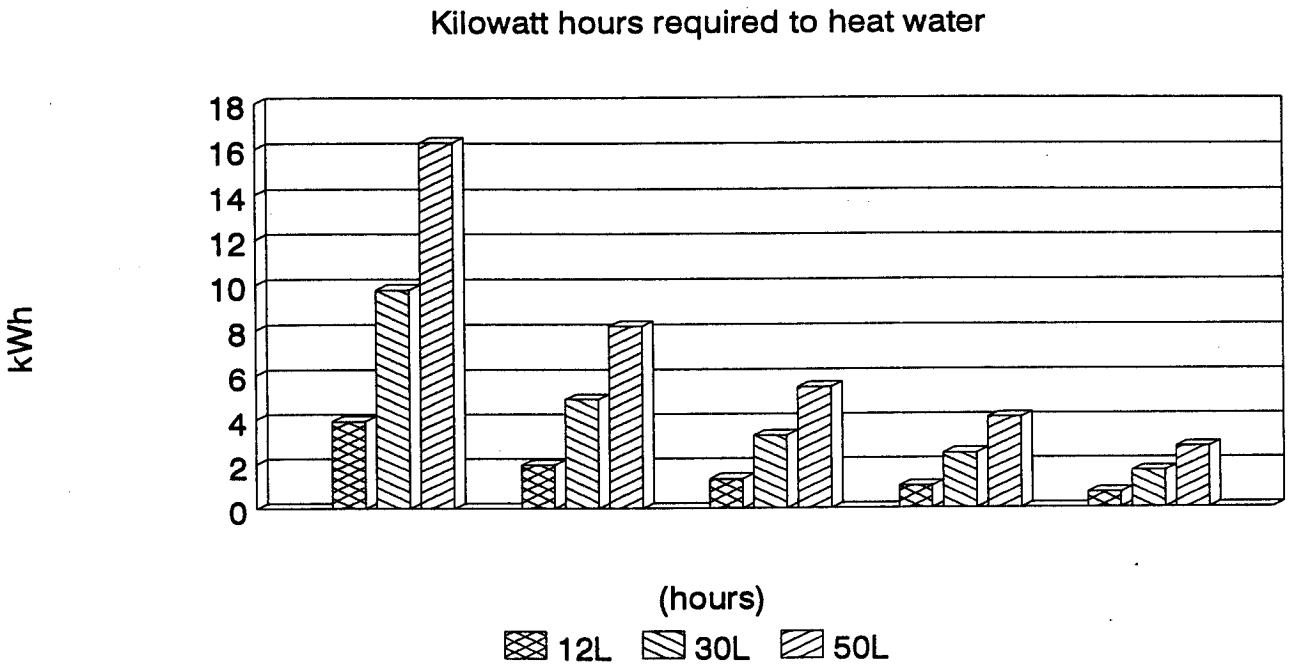
$$kWh = \frac{Qw}{3412} + \frac{Ls}{1000}$$

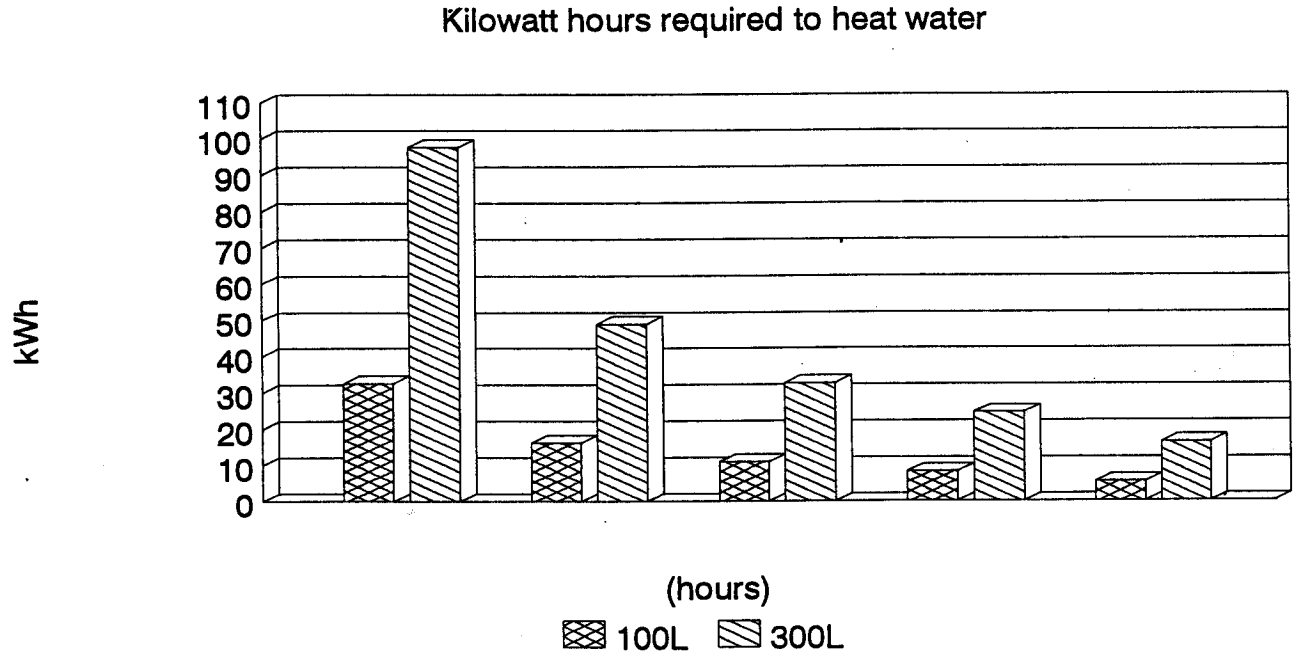
Qw = 11,020 BTU
Ls = 540 watts

Operating capacity required (kW) : 4.8

Tank heigth (ft): 1.5
Tank diameter (ft): 1.5
Tank weight (lbs): 45
Starting Temp (oF) : 151 (pre–heated)
High end Temp (oF): 251

Figure 12. Kilowatt hours required to heat water. See Table 8.





APPENDIX

NifTAL-Developed Computer Programs

Custom computer programs are available to support the financial and technical design of an MPU facility.

FAIME (Financial Analysis for Inoculant Manufacturing Enterprises)

FAIME analyzes strategies for allocating resources for start-up and operations based on expected production requirements. Key indicators of financial success are compiled and presented for review. The user may adjust figures and observe the effects of these changes on the indicators of financial success.

CAPACITY - (UNITS, TIME, COST)

UNITS, TIME, and COST are inter-active modules in single program entitled **CAPACITY.** Starting with UNITS, each module builds on the previous one to explore production capacities, fermentor units requirements, production times, and fermentor costs for expected production demands.

UNITS defines the number and volume of fermentors needed for specific inoculant requirements. Production time is also computed.

TIME defines personnel requirements to inject a known volume of broth into the carrier material. A number of variables allows modeling of production mode and scale.

COST compares the costs of the number of fermentors required to satisfy expected production demands in two production modes--sterile and non-sterile.

OUTPUT compares the two.

MPNES - Most Probable Enumeration System

MPNES provides rhizobial population estimates based on the results of MPN plant infection tests.

ACCLAIM - ACCounting for Laboratories by Automation and Information Management

ACCLAIM has three sections:

A) Database of NifTAL's Microbial Resource Center (MIRCEN) collection of rhizobia.

B) Recommended rhizobial strains for grain, tree, and pasture legumes.

C) Seed weights and inoculant application rates for selected legumes.

HEAT UP

HEAT UP allows the user to calculate kilowatt requirements for raising water temperature over a specified range.

Hardware Requirements

All programs can be installed on your computer providing you have the following hardware requirements.

1. DOS system, version 2.0 or higher
2. 512K RAM
3. 3.5" floppy drive
4. Recommended: Hard disk installation with 1 Mb free

The Use of Sterile or Non-Sterile Inoculant Carriers: Which System is Best for You?

Inoculant Carriers : Inoculant carriers are a critical factor in the inoculant production system. After growing the rhizobial broth in fermentors, the rhizobia are mixed with an inoculant carrier material. The quality of the carrier depends on the number of rhizobia it can support. Good carriers can support one billion (109) cells per gram when the inoculant is fresh, and maintain 100 million (108) six months after production.

Carriers are most often finely ground peat, which has been proven as an industry standard. Other substances such as lignite, filter mud, charcoal, soil, and other locally available materials have also been used. The materials are processed so that they can support rhizobial growth and survival. They must also be fine enough to stick to the seed. For example, indigenous peat must be mined, dried, ground, and sieved. The resultant material is then limed before it is a can be used as a carrier to support rhizobia.

Inoculant production systems use either sterile or non-sterile carriers. Each mode of production has its own capital, material, and processing requirements. The advantages and disadvantages are presented below.

Production with Sterile Carriers : The use of sterile carriers means that the carrier material must be prepackaged and sterilized prior to incorporation of rhizobial broth. The inoculant producer must package and sterilize local materials on site, or purchase ready-to-use sterile (gamma-irradiated) peat packets from commercial suppliers. Small scale producers may be able to steam sterilize their own materials, but this heat sterilization may cause adverse changes to the carrier or packaging.

The primary advantage to using sterile carriers is that the Broth Dilution Technique can be used. With this dilution technique, full strength rhizobial broth from the fermentor is diluted with sterile water. This diluted culture is then injected into the individual bags of peat, and allowed to "cure" for 5 to 7 days at 24 - 28 °C. There, the rhizobia can use the nutrients in the carrier to regain its original "full-strength" population level. Inoculants produced with sterile carriers have a shelf life of four to six months if kept cool, and six to nine months under refrigeration.

Capital investments for fermentation systems are reduced when using the dilution technique. Dilution factors of 1:50 and 1:100 are often used in production. This dilution reduces the required fermentation capacity by the same factors. Quality control of the inoculants is also simplified. Because there are no other organisms in the carrier, the rhizobia can be counted directly using common microbiological techniques.

The major disadvantage to using sterile carriers is that sterilization facilities must be found, or pre-packaged, presterilized peat must be purchased from suppliers in the US or Australia. While this prepared material is convenient to use, transportation costs and foreign currency requirements for importing the peat packets may be obstacles.

Production with Non-Sterile Carriers : Introducing the rhizobial broth to non-sterile carriers can be as simple as pouring the broth into an open bin with a known quantity of carrier, and thoroughly mixing by hand or hoes. The mixture is then packaged. The curing requirements are the same as for sterile carriers. After curing, the inoculants must be kept under refrigeration to preserve the rhizobial population. Shelf life of non-sterile materials is much shorter than with sterile carriers because of the presence of other organisms in the inoculant. Without refrigeration, the quality of the product is questionable after a few weeks.

Advantages to using non-sterile production methods include the ease of physical processing of large amounts of material. Indigenous carriers can be used, and the dependence on imported, prepackaged carriers or sterilization facilities is eliminated. Requirements for stringent aseptic handling techniques are reduced, therefore the need for specially trained staff is also minimized.

The use of non-sterile carriers requires a larger fermentation capacity for producing the rhizobial broth culture because the dilution technique cannot be used. A comparatively larger inventory of supplies and chemicals is also needed. Because the product has a relatively short storage life, an stringent production schedule must be maintained to meet the demands of seasonal planters. In addition, quality control measures to enumerate the rhizobia in the peat will depend on plant infection tests, and the appropriate facilities to conduct the tests. Results may take up to four to six weeks.

Sterile Carrier System	
Advantages	Disadvantages
■ Used in small to medium scale operations. ■ Dilution of broth means reduced investment in fermentation vessels, media, and supplies to produce rhizobial broth. ■ High quality product with long shelf life. ■ Production schedule not as rigid due to longer shelf life. ■ Ease of quality control measures. ■ Pre-packaged, pre-sterilized material is convenient to use.	■ Requires sterile carrier. Producer must sterilize indigenous materials or purchase pre-sterilized, pre-packaged materials. Disadvantages may include expenses and problems associated with importing this prepared material. ■ Need method for aseptic dilution with sterile water. ■ More intensive handling. Broth must be introduced and mixed into individual packets. ■ Aseptic handling means requirements for trained personnel higher.

Non-Sterile Carrier System	
Advantages	Disadvantages
■ Used in medium to large scale production systems. ■ Incorporating rhizobial broth into carrier material is a simple process. ■ Materials handled in bulk. ■ Indigenous materials can be used. ■ Processing of indigenous materials reduced since sterilization not necessary. ■ Need for specially trained staff reduced.	■ Broth dilution technique cannot be used and larger fermentation capacity required. ■ Need more supplies for producing large quantities of broth culture. ■ Shorter shelf life of product. ■ Rigid production schedule due to short shelf life. ■ Refrigeration of product to extend shelf life is expensive. ■ Quality control more difficult. Enumeration of rhizobia requires plant infection tests and facilities for conducting test.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1982, p. 330–333
0099-2240/82/080330-04$02.00/0

Vol. 44, No. 2

Dilution of Liquid *Rhizobium* Cultures To Increase Production Capacity of Inoculant Plants†

PADMANABHAN SOMASEGARAN* AND JAKE HALLIDAY

NifTAL Project, Department of Agronomy and Soil Science, University of Hawaii, Honolulu, Hawaii 96822

Received 16 February 1982/Accepted 27 April 1982

Experiments were undertaken to test whether peat-based legume seed inoculants, which are prepared with liquid cultures that have been deliberately diluted, can attain and sustain acceptable numbers of viable rhizobia. Liquid cultures of *Rhizobium japonicum* and *Rhizobium phaseoli* were diluted to give 10^8, 10^7, or 10^6 cells per ml, using either deionized water, quarter-strength yeast-mannitol broth, yeast-sucrose broth, or yeast-water. The variously diluted cultures were incorporated into gamma-irradiated peat, and the numbers of viable rhizobia were determined at intervals. In all of the inoculant formulations, the numbers of rhizobia reached similarly high ceiling values by 1 week after incorporation, irrespective not only of the number of cells added initially but also of the nature of the diluent. During week 1 of growth, similar multiplication patterns of the diluted liquid cultures were observed in two different peats. Numbers of rhizobia surviving in the various inoculant formulations were not markedly different after 6 months of storage at 28°C. The method of inoculant preparation did not affect the nitrogen fixation effectiveness of the *Rhizobium* strains.

Leguminous crops can be self-sufficient for all or part of their nitrogen requirements when their roots are nodulated with an effective nitrogen-fixing strain of *Rhizobium*. Some legumes nodulate only with specific strains of *Rhizobium* or may form fully effective nitrogen-fixing associations with a few of the array of strains that can nodulate them. Therefore, seed or soil inoculation with preselected rhizobia is practiced to ensure nodulation and nitrogen fixation in grain and forage legumes. Production of inoculants involves culture of *Rhizobium* on a large scale and incorporation into a carrier material, usually finely milled peat. In addition to their nutritional requirements, rhizobia need stirring and aeration to reach a high population density in liquid culture. A basal nutrient medium with mannitol as the carbon source and including yeast extract is the standard formulation for culturing rhizobia (11). Stirring and aeration are readily achieved in Erlenmeyer flasks plugged with cotton wool and mounted on a rotary shaker. Rhizobia are microaerophilic (12), and oxygen requirements are satisfied at the minimum speed necessary to prevent sedimentation of cells in the flasks. Because of technical difficulties in the development of large-scale, continuously operating shakers and the impracticality of autoclaving very large flasks, the shaker-flask system has been considered suitable only for production of inoculants on a laboratory scale. Large-scale production has involved the use of fermentors, commonly of 1,000 to 2,000 liters, with special provisions for stirring and aeration (2).

Such fermentors are expensive to acquire and maintain and costly to install and operate. They are prone to contamination because of the difficulty in sterilizing all access lines and ensuring contaminant-free aeration throughout culture growth. The need for skilled operators contributes to the high operating cost of such production systems. These factors have not prevented establishment of viable inoculant production enterprises in industrialized nations. In developing countries, however, they are major disincentives for public and private entities contemplating production of legume seed inoculants.

It is known that *Rhizobium meliloti, Rhizobium trifolii*, and a cowpea *Rhizobium* reach similar ceiling populations in peat after 3 weeks of incubation, when liquid cultures of different ages (which, therefore, contain different numbers of viable cells) are used (8). So far, this phenomenon has not been recognized as a way of greatly increasing the production output of shaker-flask-based inoculant plants.

This research was undertaken to test whether peat-based legume seed inoculants prepared with liquid cultures that have been deliberately

† Journal series no. 2689 of the Hawaii Institute of Tropical Agriculture and Human Resources.

TABLE 1. Influence of initial inoculum levels on subsequent growth and survival of *R. japonicum* USDA 110 and *R. phaseoli* TAL 182 in Badenoch peat incubated at 28°C

Approx level of diluted liquid culture (log_{10} viable cells per ml)[a]	Log_{10} viable cells per g of moist peat after indicated time (days):							
	R. japonicum				*R. phaseoli*			
	0	7	28	70	0	7	28	70
6	5.42	9.96[b]	9.98	9.83	4.95	9.72[b]	9.97	10.07
7	6.31	10.06[b]	9.98	9.99	6.25	9.87[b]	9.91	9.63
8	7.45	10.18[b]	10.03	9.77	7.43	10.00[b]	9.85	9.69

[a] YM broth (25%) was used as diluent.

[b] The difference in population density between time 0 and 1 week for each inoculum level was significant ($P \leq 0.01$). Other differences were not significant.

diluted can attain and sustain acceptable numbers of viable rhizobia.

MATERIALS AND METHODS

Bacteria. *Rhizobium japonicum* USDA 110 and *Rhizobium phaseoli* TAL 182 were obtained as desiccated ceramic-bead cultures (7) from the NifTAL *Rhizobium* germ plasm resource.

Culture medium. Yeast-mannitol (YM) medium was prepared as described by Vincent (11), with 0.5 g of yeast extract (Difco Laboratories, Detroit, Mich.) per liter substituted for yeast-water.

Diluents. Deionized water, quarter-strength YM medium, quarter-strength yeast-sucrose medium (prepared by substituting sucrose for mannitol in the YM medium), and quarter-strength yeast-water medium (mannitol was omitted from YM medium) were used for diluting liquid cultures of *Rhizobium* before incorporation in peat. Diluents were autoclaved at 121°C at 15 lb/in^2 for 30 min.

Peat. Finely milled Badenoch peat (pH 6.8, without neutralizing with calcium carbonate) was obtained prepackaged and sterilized by gamma irradiation in 50-g polyethylene bags from Agricultural Laboratories, Regents Park, New South Wales, Australia. Milled Wisconsin peat (pH 4.5 to 5.0) was obtained in bulk from Nitragin Co., Milwaukee, Wis. The Wisconsin peat was adjusted to pH 6.5 to 6.8 by mixing with 5% (wt/wt) food grade-precipitated calcium carbonate (J. T. Baker Chemical Co., Phillipsburg, N.J.). It was then packaged in 50-g lots and gamma irradiated (5 Mrad). The polyethylene bags had a thickness of 38 μm for both peats. The moisture content of the peats was 8 to 10%.

Preparation of inoculants. Rhizobia were cultured in batches of 100 ml of YM medium in 250-ml Erlenmeyer flasks on a shaker platform rotating at 100 rpm. Late log-phase cultures with approximately 1×10^9 to 2×10^9 viable rhizobia per ml were serially diluted with each of the four different diluents to give 10^8, 10^7, or 10^6 rhizobia per ml. A sample of approximately 40 ml of diluted culture was injected aseptically into each bag of peat, the exact volume being that which gave a moisture content of 50% in each case. Each inoculant formulation was replicated three times. All bags were massaged thoroughly to mix their contents and incubated at 28°C throughout the investigation.

Counting rhizobia. A 1.0-g sample from the inoculant in each package was transferred aseptically into 99 ml of quarter-strength YM medium in a milk dilution bottle. After 15 min of agitation on a wrist-action shaker, serial dilutions were performed. Using the drop-plate method of Miles and Misra (6), 30-μl drops from each dilution in the series were spotted on one set of YM agar plates containing 25 mg of Congo red per liter and on a second set of plates containing 1.25 mg of brilliant green per liter. (Both Congo red and brilliant green were incorporated in the YM agar to recognize rhizobia should contamination occur. Brilliant green suppresses non-rhizobia, and therefore it is a useful check on Congo red, especially with bacteria not absorbing Congo red.) Plates were incubated at 28°C and counted after 3 days for strain TAL 182 and after 7 days for strain USDA 110.

Nitrogen-fixing effectiveness of inoculants. Inoculants prepared in the various formulations were tested for effectiveness on their respective host plants in Leonard jars (5) containing nitrogen-free nutrient solution (1). Surface-sterilized and pregerminated seeds were inoculated at sowing with 2 ml of a peat inoculum prepared by suspending 1 g of peat inoculant in 99 ml of quarter-strength YM medium. Test samples were taken from inoculants aged for 3 and 6 months.

Immunofluorescence. Immunofluorescent examination was done only on cultures from the short-term experiment, which was designed to confirm that the rapid population increases observed during week 1 of growth were caused by the inoculant strains. Fluorescein isothiocyanate-conjugated sera of strains TAL 182 and USDA 110 were obtained from the NifTAL serum bank. Smears were prepared from colonies picked from plates (from 0-, 4-, and 7-day counts) and stained by the method of Schmidt et al. (10). At least 50% of the colonies resulting from a single drop (20 to 30 colonies per drop) were examined for contaminants by epifluorescence alone or in combination with the phase-contrast system. When colonies were too numerous to pick, a composite of the growth was made. A sample smear from the composite was then stained and examined.

RESULTS AND DISCUSSION

R. japonicum USDA 110 and *R. phaseoli* TAL 182 attained population densities of close to 10^{10} viable cells per g of moist peat by 1 week after incorporation, even though the initial numbers in the diluted liquid culture varied in the range 10^6 to 10^8 rhizobia per ml (Table 1). Numbers of viable rhizobia were sustained well above 10^9 for

TABLE 2. Effect of different diluents on growth and survival of *R. japonicum* USDA 110 in Badenoch peat during long-term storage at 28°C

Diluent (concn)	Log_{10} viable cells per g of moist peat after incorporation for (days):								LSD ($P \leq 0.01$)[a]
	0	14	30	60	90	120	150	180	
Sterile, deionized water	4.32	10.01	9.83	9.73	9.43	8.65	9.08	7.89	0.31
YM medium (25%)	4.41	10.06	9.96	9.93	9.60	8.74	9.23	8.03	0.45
Yeast-water medium (25%)	4.37	9.81	9.88	9.77	9.63	8.81	9.17	7.72	0.32
Yeast-sucrose medium (25%)	4.53	9.94	9.98	9.91	9.46	9.08	9.31	7.94	0.23

[a] Zero-time viable counts were not included in the analysis. LSD, Least significant difference.

10 weeks. All multiplications were more rapid than those in the experiments described by Roughley (8). The results indicated that a 1,000-fold dilution (from 10^9 to 10^6) of the liquid culture is acceptable and suggest that even greater dilutions might be possible without affecting the final population density attained in the inoculants.

The tests with a range of diluents revealed that inoculants with high populations of rhizobia could be prepared with each diluent (Tables 2 and 3). Analysis of the data showed that the four diluents did not differ significantly in their abilities to support growth and survival of both strains. Although no measurements were made to confirm the moisture contents of the inoculants after long-term storage, significant moisture loss was indicated by the dry appearance of the peat. A gradual and significant decline in population increasing with storage time was observed which may be attributed to the effects of desiccation of the inoculants. All three strains of rhizobia were adversely affected during storage by a low moisture content in the peat (9). Numbers of viable rhizobia surviving in each formulation after 6 months of storage were equal or very close to the accepted standard of 10^8 cells per g of moist peat (3). The high populations of rhizobia sustained in the inoculants in which water was used as a diluent indicated that the carry-over of nutrients in the diluted YM medium and in the peat itself could support high populations of rhizobia during the 6-month shelf life. The chemical composition of the peat (3) may have an intrinsic nutritional value for rhizobia.

The nitrogen-fixing effectiveness of the rhizobia in the various inoculant formulations was confirmed to have been maintained. The appropriate host plants inoculated with samples from 3- and 6-month-old inoculants produced healthy, green plants. Roots were well nodulated, and nodule sections showed pink-to-red interiors.

Based on the typical mean generation times for fast- and slow-growing rhizobia given by Vincent (12), the rapid increases in population observed in all inoculants after incubation for only 1 week are theoretically possible. Nevertheless, a follow-up experiment was warranted to monitor cell multiplication at daily intervals after incorporation of cultures in the peat carrier. In this short-term experiment, inoculants of each strain were prepared as described above. Both Badenoch and Wisconsin peats were investigated.

Cells of each of the two strains multiplied rapidly (Fig. 1) in both Badenoch and Wisconsin peats. Maximum populations were attained after 4 to 5 and 7 days by undiluted and 1,000-fold-diluted cultures, respectively. The inoculants were confirmed to be free of contaminants by plating and typing colonies by immunofluorescence with the specific antisera. The mean generation times for *R. phaseoli* were 2.7 and 2.8 h in Badenoch and Wisconsin peats, respectively. *R. japonicum* had a mean generation time (7.0 h)

TABLE 3. Effect of different diluents on growth and survival of *R. phaseoli* TAL 182 in Badenoch peat during long-term storage at 28°C

Diluent (concn)	Log_{10} viable cells per g of moist peat after incorporation for (days):								LSD ($P \leq 0.01$)[a]
	0	14	30	60	90	120	150	180	
Sterilized, deionized water	4.46	9.75	9.69	9.36	8.89	8.67	8.28	8.29	0.43
YM medium (25%)	4.39	9.98	9.75	9.58	8.93	8.47	8.42	8.04	0.46
Yeast-water medium (25%)	4.43	9.69	9.53	9.44	8.81	8.60	8.24	8.05	0.39
Yeast-sucrose medium (25%)	4.31	9.98	9.79	9.60	9.02	8.67	8.50	8.22	0.38

[a] Zero-time viable counts were not included in the analysis. LSD, Least significant difference.

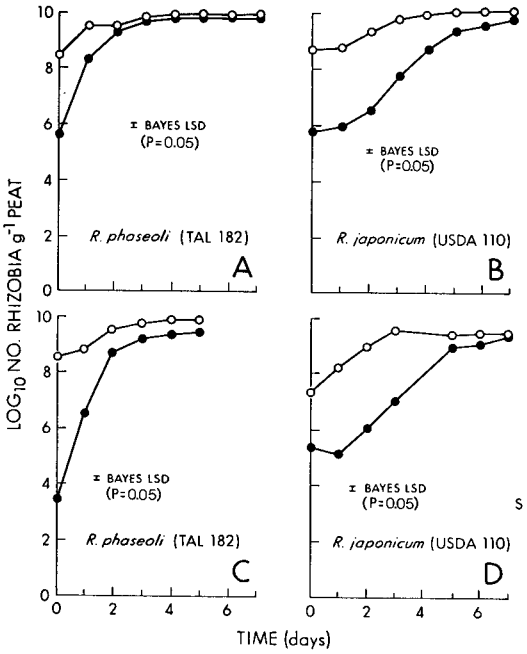


FIG. 1. Multiplication of *Rhizobium* in sterilized Badenoch (A and B) and Wisconsin (C and D) peats after incorporation of full-strength (○) and diluted (●) liquid culture. Diluent was 25% YM broth. The full-strength culture was diluted 1,000-fold for the diluted liquid culture treatment. Each treatment was set up in triplicate. BAYES LSD (least significant difference): k = 100.

in the Badenoch peat that was slightly shorter than the 7.7-h value found for Wisconsin peat. These values compare well with the expected mean generation times for fast- and slow-growing rhizobia in liquid culture (12).

The results showed that, for these representative fast- and slow-growing strains of *Rhizobium*, the continued and rapid multiplication of rhizobia after incorporation in the peats tested can be relied upon to produce acceptable inoculants from diluted cultures. The method may also be extended to strains of rhizobia which have specific requirements (4) of a fairly expensive carbon source, especially some strains of soybean and lupine rhizobia which prefer arabinose and other pentoses. Such organisms may be grown in small volumes before dilution. These advantages offer the opportunity to devise large-output inoculant production systems in which rhizobia are cultured simply and inexpensively in flasks or shakers.

Although the method described above was performed with irradiated peat, initial studies have shown that this method can be applied to steam-sterilized peat. The data from these experiments will be presented elsewhere.

ACKNOWLEDGMENTS

This work was supported by the United States Agency for International Development under contract AID/ta-C-1207.

We acknowledge the skilled technical assistance of B. Martin and P. Nakao.

LITERATURE CITED

1. **Broughton, W. J., and M. J. Dilworth.** 1971. Control of leghaemoglobin synthesis in snake beans. Biochem. J. **125**:1075–1080.
2. **Burton, J. C.** 1967. *Rhizobium* culture and use, p. 1–33. *In* H. J. Peppler (ed.), Microbial technology. Van Nostrand Reinhold, New York.
3. **Date, R. A., and R. J. Roughley.** 1977. Preparation of legume seed inoculants, p. 243–275. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on nitrogen fixation. Section IV: agronomy and ecology. John Wiley and Sons, Inc., New York.
4. **Graham, P. H., and C. A. Parker.** 1964. Diagnostic features in the characterization of the root-nodule bacteria of the legumes. Plant Soil **20**:383–396.
5. **Leonard, L. T.** 1944. Method of testing bacterial cultures and results of tests of commercial inoculants, 1943. U.S. Department of Agriculture Circular no. 703. U.S. Department of Agriculture, Washington, D.C.
6. **Miles, A. A., and S. S. Misra.** 1938. The estimation of the bactericidal power of blood. J. Hyg. **38**:732–749.
7. **Norris, D. O.** 1963. A porcelain bead method for storing *Rhizobium*. J. Exp. Agric. **31**:255–258.
8. **Roughley, R. J.** 1968. Some factors influencing the growth and survival of root nodule bacteria in peat culture. J. Appl. Bacteriol. **31**:259–265.
9. **Roughley, R. J., and J. M. Vincent.** 1967. Growth and survival of *Rhizobium* spp. in peat culture. J. Appl. Bacteriol. **30**:362–376.
10. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluorescent-antibody approach to the study of rhizobia in soil. J. Bacteriol. **95**:1987–1992.
11. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. International Biological Programme handbook no. 15. Blackwell Scientific Publications, Oxford, England.
12. **Vincent, J. M.** 1974. Root-nodule symbioses with *Rhizobium*, p. 265–341. *In* A. Quispel (ed.), The biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1985, p. 398–405
0099-2240/85/080398-08$02.00/0

Vol. 50, No. 2

Inoculant Production with Diluted Liquid Cultures of *Rhizobium* spp. and Autoclaved Peat: Evaluation of Diluents, *Rhizobium* spp., Peats, Sterility Requirements, Storage, and Plant Effectiveness

P. SOMASEGARAN

NifTAL Project, Department of Agronomy and Soil Science, University of Hawaii, Honolulu, Hawaii 96822

Received 13 November 1984/Accepted 29 April 1985

Fully grown broth cultures of various fast- and slow-growing rhizobia were deliberately diluted with various diluents before their aseptic incorporation into autoclaved peat in polypropylene bags (aseptic method) or mixed with the peat autoclaved in trays (tray method). In a factorial experiment with the aseptic method, autoclaved and irradiated peat samples from five countries were used to prepare inoculants with water-diluted cultures of three *Rhizobium* spp. When distilled water was used as the diluent, the multiplication and survival of rhizobia in the peat was similar to that with diluents having a high nutrient status when the aseptic method was used. In the factorial experiment, the mean viable counts per gram of inoculant were log 9.23 (strain TAL 102) > log 8.92 (strain TAL 82) > log 7.89 (strain TAL 182) after 24 weeks of storage at 28°C. The peat from Argentina was the most superior for the three *Rhizobium* spp., with a mean viable count of log 9.0 per g at the end of the storage period. The quality of inoculants produced with diluted cultures was significantly ($P = 0.05$) better with irradiated than with autoclaved peat, as shown from the factorial experiment. With the tray method, rhizobia in cultures diluted 1,000-fold or less multiplied and stored satisfactorily in the presence of postinoculation contaminants, as determined by plate counts, membrane filter immunofluorescence, and plant infection procedures. All strains of rhizobia used in both the methods showed various degrees of population decline in the inoculants when stored at 28°C. Fast- and slow-growing rhizobia in matured inoculants produced by the two methods showed significant ($P < 0.01$) decline in viability when stored at 4°C, whereas the viability of some strains increased significantly ($P < 0.01$) at the same temperature. The plant effectiveness of inoculants produced with diluted cultures and autoclaved peat did not differ significantly from that of inoculants produced with undiluted cultures and gamma-irradiated peat.

In the usual method of peat-based inoculant production, rhizobia are multiplied in fermenters to reach maximal populations before being added to the peat. In the pure-culture or aseptic technique, a definite volume (30 to 50% of the total final weight) of a fully grown culture (10^9 cells per ml) is added aseptically to a relatively dry peat sample (8 to 10% moisture) which has been presterilized in polyethylene bags by gamma irradiation. The added rhizobia multiply further to reach maximal populations since the peat was presterilized, but minimal to no multiplication may take place if the peat was not sterilized. General experience has shown that, with the exception of a few strains that can reach a population of 10^{10} cells per g of peat inoculant, most strains rarely exceed 5×10^9 cells per g in irradiated peat (12). In heat-treated sedge peat used in the United States, the number of added rhizobia reaches 2.5×10^9 to 3.0×10^9 cells per g of inoculant (4). The choice of technique used depends very much on the scale of production, with the nonsterile peat method being the choice of most commerical producers. However, in Australia, all peat used in commerical production is gamma irradiated in polyethylene packages (13, 20). The production capacity of both techniques is directly proportional to the volume of liquid cultures that can be produced, and this in turn is limited by the size of the fermenter. The problem is further magnified when liquid cultures for several species of legumes are needed.

In developing countries, where there is a great need for legume inoculants, numerous problems are associated with inoculant production, e.g., in Guyana a frequent problem is the availability of a suitably sized and low-cost fermenter (11).

The high costs and operational problems associated with large-scale fermenters led to an investigation of the potential of using diluted liquid cultures of *Rhizobium* spp. for inoculant production with irradiated peat (16). In developing countries, costly gamma-irradiation facilities are not always feasible for peat sterilization, but steam sterilization is a realistic alternative. Steam sterilization has been used in the sterilization of peat for inoculant production with undiluted cultures (9, 19, 22). The use of autoclaved peat and diluted liquid cultures of *Rhizobium* spp. has not been investigated as a new and potential method for inoculant production. This report characterizes inoculants produced by the dilution method.

MATERIALS AND METHODS

Rhizobia. The various *Rhizobium* spp. (Table 1) used in this investigation were obtained from the NifTAL *Rhizobium* germ plasm resource. Cultures were maintained on yeast mannitol agar (YMA) as described by Vincent (23) and checked for purity by the fluorescent-antibody technique (15).

Diluents. Various formulations of diluents were used to dilute late-log-phase liquid cultures to investigate the influence of the nutrient status of the diluent on the multiplication and survival of the rhizobia in the peat. The preparation of these diluents was described previously (16), and they are listed in Table 2. Only *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 were used to evaluate the influence of diluents.

In subsequent experiments with other strains of rhizobia, only sterile, deionized water was used to dilute the cultures.

TABLE 1. Sources of *Rhizobium* cultures

Designations[a] of *Rhizobium* spp.		Cross-inoculation group	Parent host
TAL	Original		
82	TAL 82	*Rhizobium* sp.	*Leucaena leucocephala*
102	USDA 110	*R. japonicum*	*Glycine max*
169	Nitragin 176A22	*Rhizobium* sp.	*Vigna unguiculata*
182	TAL 182	*R. phaseoli*	*Phaseolus vulgaris*
379	USDA 136b; CB1809	*R. japonicum*	*Glycine max*
380	SU 47	*R. meliloti*	*Medicago sativa*
620	ICRISAT 3889	*Rhizobium* sp.	*Cicer arietinum*
651	UMKL 44	*Rhizobium* sp.	*Calopogonium mucunoides*
1376	C-34	*R. phaseoli*	*Phaseolus vulgaris*

[a] Labratory sources were as follows: TAL, NifTAL Project, University of Hawaii, Honolulu; USDA, U.S. Department of Agriculture, Beltsville, Md.; Nitragin, Nitragin Co., Milwaukee, Wis.; SU, University of Sydney, Sydney, Australia; ICRISAT, International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India; UMKL, University of Malaya, Kuala Lumpur, W. Malaysia; C, Centro de Energia Nuclear na Agriculture, Piracicaba, Brazil.

Sterilization of the deionized water was achieved by autoclaving or by filtration through a 0.2-μm Ultipor disposable self-contained membrane filter unit (Pall Corporation, Cortland, N.Y.). This filter was reused several times after sterilization by autoclaving. Pressure for filtration was provided by a liquid circulation pump (Flotec Inc., Norwalk, Calif.).

Presterilization of peat in plastic bags. Finely milled Demilco peat (pH 4.5 to 5.0; moisture content, 8 to 10%) was purchased from the Nitragin Co., Milwaukee, Wis. The peat was mixed with food-grade precipitated calcium carbonate (J. T. Baker Chemical Co., Phillipsburg, N.J.) at a ratio of 50 g of peat to 2.5 g of calcium carbonate to give a final pH of 6.5 to 6.8. The peat was then packaged in 50-g lots in thin (38-μm) polyethylene bags, heat sealed, and gamma irradiated (5 Mrad).

Similar quantities of peat were placed in high-density (38 μm) autoclavable polypropylene bags (Sealcraft Packaging Corp., Milwaukee, Wis.). The open end of each bag was folded back to produce a narrow flap, 4 cm in width, which was held in place by two no. 1 paper clips. The bags were arranged in rectangular wire baskets with sufficient space for steam circulation between bags. Before placement of the bags, the wire baskets were lined with paper towels on the sides and bottom to prevent metal-plastic contact during autoclaving. The peat was sterilized for 60 min at 121°C and at 1.05 kg/cm^2. The bags were removed for sealing after overnight cooling in the autoclave. They were sealed in a laminar flow hood. The two paper clips were removed, and a seal was made along the crease of the flap.

Peat from Mexico (FERTIMEX, Anaxagoras 25, Mexico City) Argentina (Nitrasoil Argentina S.A., Buenos Aires), Spain (Instituto Nacional de Investigaciones Agrarias, San Jose de la Rinconada), and Peru (Universidad Nacional de Cajamarca, Cajamarca) was packaged and sterilized by irradiation and autoclaving as with the Demilco peat from the United States. These imported peat samples did not require the addition of the calcium carbonate, as their pHs were 6 to 7.0.

Presterilization of peat in trays. A 1-kg portion of pH-amended Demilco peat was placed in a heavy-duty autoclavable polypropylene tray (18 by 18 by 6 in) and spread out to give a layer of even thickness. An aluminum foil cover was placed over the tray. The peat was autoclaved at 121°C and 1.05 kg/cm^2 for 60 min and left to cool overnight in the autoclave. The aluminum foil cover was left in place until removal just before inoculant preparation.

Preparation of inoculants and storage. Late-log-phase cultures were obtained in YM medium and diluted to the required levels with the various diluents.

To examine the effect of sterility of the peat on the multiplication and survival of the rhizobia, the peat samples were inoculated with the diluted cultures under aseptic or septic conditions. All inoculants were prepared with single strains.

For the aseptic method, presterilized peat samples in polyethylene or polypropylene bags were aseptically inoculated with 40 ml of the diluted culture (50- to 100-fold dilution) with a sterile, plastic syringe fitted with a hypodermic needle (8). The bags were thoroughly kneaded to ensure absorption of the liquid culture into the peat. Inoculants were stored (incubated) at 28°C. To study survival under low-temperature storage conditions, inoculants were sampled for enumeration and immediately transferred to a refrigerator (4°C). The same inoculants were reenumerated after a fixed period of storage at 4°C.

For the septic method (referred to as the tray method henceforth), peat inoculation was done in the open laboratory environment. The diluted culture (approximately 50- to

TABLE 2. Shelf life of inoculants prepared from autoclaved peat and diluted liquid cultures of *R. phaseoli* (TAL 182) and *R. japonicum* (TAL 102)

Strain and diluent	Log_{10} (no. of rhizobia/g of moist peat)[a] after storage for (weeks):				
	0	2 (22)[b]	8	16	24
R. phaseoli					
Sterile, deoinized water	4.41	9.18 (6.47)	8.08	7.53	7.50
25% yeast-mannitol broth	4.85	9.40 (5.68)	8.14	7.87	7.60
25% yeast-water	4.92	9.09 (3.79)	8.08	7.64	7.37
25% yeast sucrose broth	4.81	9.54 (5.75)	7.99	7.73	7.47
R. japonicum					
Sterile, deoinized water	4.68	8.11 (9.03)	9.32	9.29	9.18
25% yeast mannitol broth	4.76	7.93 (9.20)	9.26	9.16	9.10
25% yeast-water	4.75	8.02 (9.19)	9.35	9.13	9.26
25% yeast sucrose broth	4.67	8.54 (9.22)	9.36	9.40	9.27

[a] Mean of three replications.
[b] Inoculant was stored at 28°C for 2 weeks and then at 4°C for 22 weeks. Values in parentheses are plate counts after storage at 4°C.

100-fold dilution) was mixed at a rate of 35 ml per 50 g of peat. Mixing was done by hand; sanitized, disposable polyethylene gloves (VWR Scientific, San Francisco, Calif.) were worn throughout the mixing process. Immediately after being mixed, 100-g quantities of the inoculant were packaged in clean, previously unused polyethylene bags and heat sealed. The survival of rhizobia in these inoculants was compared with that of inoculants of the same strains prepared with undiluted culture inoculated aseptically into bags of irradiated peat as described for the aseptic method. The inoculants prepared by this tray method were also tested for storage characteristics at 4°C.

Enumeration of rhizobia by plate counts and plant infection (MPN) technique. Serially diluted samples of the inoculants were plated on Congo red YMA (CRYMA) and brilliant green YMA (BGYMA) media (16) by the drop and spread plate methods as described previously (8). The drop plate method was routinely used for enumerating pure cultures of rhizobia in inoculants prepared from presterilized peat. Rhizobia in inoculants prepared by the tray method were enumerated by the spread plate (CRYMA and BGYMA) method and the standard plant infection (most probable number [MPN]) technique (23) with plastic growth pouches (24) or in enclosed seedling agar tubes (23). Tenfold serial dilutions and four replications per dilution were used for MPN counts. The following host plants were used for the MPN counts: *Glycine max* cv. Davis for *R. japonicum* TAL 379, a small, white-seeded commercial variety of *Phaseolus vulgaris* for *R. phaseoli* TAL 182, *Leucaena leucocephala* K8 for *Rhizobium* sp. strain TAL 82, *Medicago sativa* for *R. meliloti* TAL 380, and *Macroptilium atropurpureum* var. Siratro for *Rhizobium* spp. strain TAL 169 and TAL 651. Surface-sterilized seeds were pregerminated on water agar plates. Seedlings of *Medicago sativa* and *Macrophilium atropurpureum* var. Siratro were established in seedling agar tubes, and the other hosts were in growth pouches. Uninoculated controls were included in all treatments, and plants were scored for the presence or absence of nodules at 3 weeks after inoculation.

Enumeration of rhizobia by the MFIF technique. Direct counting of the rhizobia by membrane filter immunofluorescence (MFIF) was done with polycarbonate Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) stained with irgalan black (7), since the inoculants prepared by the tray method would contain contaminants. A 5-ml sample from the 10^{-4} dilution was filtered, and the filter was treated with gelatin-rhodamine isothiocyanate conjugate (2). The direct fluorescent-antibody technique was used for staining the rhizobia (15), and 20 randomly chosen microscope fields were counted per filter at ×100 magnification. MFIF observations were made with a Zeiss standard microscope 14 with an incident light fluorescence illuminator equipped with an HBO 50-W mercury vapor light source.

Immunofluorescence examination of colonies on plates. To test the accuracy of recognition of rhizobial colonies from contaminants during plate counts, smears were made from colonies picked from CRYMA and BGYMA plates. All inoculants produced by the tray method were examined. Five colonies recognizable as those of the inoculant *Rhizobium* sp. were picked for observation from every replication at each sampling period.

Nitrogen-fixing effectiveness of inoculants. Inoculants of *R. japonicum* TAL 102, *R. phaseoli* TAL 182, and *Rhizobium* sp. strain TAL 82 prepared with diluted cultures and presterilized peat (autoclaved and irradiated) from various countries were tested for their effectiveness on their appropriate host plants. The performance of each of these inoculants was compared with that of the appropriate multistrain NifTAL inoculant which was prepared with an undiluted culture containing three strains and irradiated peat. The inoculants were tested after aging for 6 months at 28°C. Inoculation procedures and plant culture techniques were as previously described (16). Host-*Rhizobium* combinations and harvest time were as follows: *Phaseolus vulgaris* cv. Bountiful-*R. phaseoli* TAL 182 harvested at 30 days, *G. max* cv. Davies-*R. japonicum* TAL 102 harvested at 31 days, and *Leucaena leucocephala* cv. K8-*Rhizobium* sp. strain TAL 82 harvested at 48 days. At harvest, plant shoots were excised and dried at 70°C for 48 h for dry weight determination. Roots were examined for nodulation. Sample nodules were cut open, and the color of the nodule interior was noted.

TABLE 3. Shelf life of inoculants prepared from autoclaved peat and diluted liquid cultures of *Rhizobium* spp.[a]

Rhizobium spp.	Log_{10} (no. of rhizobia/g of moist peat) after storage for (weeks):				
	2 (22)[b]	3	8	16	24
Rhizobium sp. TAL 82	9.45 (ND)[c]	8.92	8.71	8.23	7.89
Rhizobium sp. TAL 169	6.26 (7.30)	8.29	9.14	8.88	8.77
R. japonicum TAL 379	9.77 (9.53)	9.72	9.51	9.31	9.16
R. meliloti TAL 380	9.62 (9.23)	9.77	9.11	8.70	8.65
Rhizobium sp. TAL 620	9.41 (9.87)	9.61	9.30	8.94	8.72
Rhizobium sp. TAL 651	9.41 (8.00)	9.45	9.22	9.01	8.89
R. phaseoli TAL 1376	9.35 (8.47)	9.08	8.10	7.67	7.67

[a] Late-log-phase cultures were diluted to 10^6 rhizobia per ml in deionized water that had been sterilized by membrane filtration.
[b] Inoculant was stored at 28°C for 2 weeks and then at 4°C for 22 weeks. Values in parentheses are plate counts after storage at 4°C.
[c] ND, Not done.

RESULTS

Evaluation of diluents. The influence of various diluents on the subsequent multiplication and long-term survival of *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 in inoculants prepared from autoclaved peat and 1,000-fold-diluted liquid cultures is shown in Table 2. The sterile, deionized water was not significantly different from the other three nutrient-enriched diluents in supporting multiplication and survival of both species. The rates of multiplication of the two species differed: maximum populations of *R. phaseoli* were counted at 2 weeks, and maximum populations of *R. japonicum* were counted at 8 weeks. Long-term storage (24 weeks at 28°C) data for *R. phaseoli* TAL 182 indicated a significant ($P < 0.001$) decline in viability, with similar trends for all four diluent treatments. Also, long-term storage of *R. phaseoli* TAL 182 at 4°C for 22 weeks significantly (paired t test, $P < 0.01$) affected survival in the peat, by a decrease of 3.88 logs. With *R. japonicum* TAL 102, long-term storage at 4°C for the same period resulted in a significant (paired t test, $P < 0.01$) increase in the population, by approximately 1.0 log.

Performance of water-diluted liquid cultures of different strains in autoclaved peat. Since sterilized, deionized water was not different from any of the nutrient-enriched diluents tested with *R. phaseoli* TAL 182 and *R. japonicum* TAL 102, seven other *Rhizobium* spp. were selected for further investigation. Inoculants were prepared with diluted liquid cultures and autoclaved peat, and the inoculant shelf life was evaluated (Table 3). An analysis of variance indicated that the growth and multiplication of the seven species differed significantly ($P < 0.01$). Except for *Rhizobium* sp. strain

TABLE 4. Compatibility of different peat samples with diluted cultures of *Rhizobium* spp. for inoculant production

Peat source	Peat presterilization	Log_{10} (no. of rhizobia/g of moist peat)[a] after storage for (weeks):								
		R. phaseoli TAL 182			*R. japonicum* TAL 102			*Rhizobium* sp. TAL 82		
		2[b]	4[b]	24[b]	2	4	24	2	4	24
Mexico	A[c]	8.61	8.58	7.69	9.00	9.20	9.13	9.02	9.02	9.01
	G[c]	9.30	9.39	7.65	9.47	9.55	9.20	9.56	9.47	9.18
Argentina	A	9.06	9.07	8.32	9.43	9.58	9.42	9.25	9.26	9.20
	G	9.51	9.70	8.56	9.67	9.86	9.62	9.88	9.70	9.25
Spain	A	8.99	9.19	7.73	9.31	9.19	9.09	9.38	9.18	9.04
	G	9.17	9.50	7.92	9.39	9.48	9.14	9.58	9.32	9.05
United States	A	8.90	8.97	8.00	9.37	9.52	9.47	9.28	9.05	8.37
	G	8.91	8.59	8.00	9.39	9.48	9.20	8.96	9.05	8.93
Peru	A	ND[d]	ND	ND	9.23	9.20	9.18	ND	ND	ND
	G				9.64	9.65	9.22			

[a] Mean of three replications.
[b] Sampling time in weeks.
[c] A, Autoclaved; G = gamma irradiated.
[d] ND, Not done.

TAL 169, which multiplied very poorly and reached maximum populations at 8 weeks, all the other rhizobia reached populations in excess of 10^9 rhizobia per g in 2 weeks. The shelf life showed similar trends, in that all various species had significant ($P < 0.01$) loss in viability when stored at 28°C for 24 weeks. However, the shelf life of the rhizobia was highly variable; e.g., *R. japonicum* TAL 379 still maintained high populations (10^9 per g) at 24 weeks, whereas *R. phaseoli* TAL 1376 and *Rhizobium* sp. strain TAL 82 populations decreased to 10^8 per g of inoculant.

Low-temperature (4°C), long-term (22 weeks) storage qualities were also evaluated with these different species (Table 3). The survival data for each strain at 4 and 28°C were analyzed for differences by a one-way analysis of variance. The differences were highly significant ($P < 0.01$) for each strain, indicating that storage at 4°C affected growth and survival in the peat. Increases in populations at 4°C were obvious with the cowpea *Rhizobium* sp. strain TAL 169 (+1.25 logs) and the chickpea *Rhizobium* sp. strain TAL 620 (+0.5 log). However, storage at 4°C affected the viability of cowpea *Rhizobium* sp. strain TAL 651 (−1.50 logs), and *R. phaseoli* TAL 1376 (−0.87 log). Also affected, but less seriously, were *R. japonicum* TAL 379 (−0.24 log) and *R. meliloti* TAL 380 (−0.49 log).

Compatibility of diverse peat samples with diluted liquid cultures of *Rhizobium* spp. The compatibility of diluted liquid cultures of *Rhizobium* spp. for inoculant production with peat from five diverse sources was evaluated in a factorial experiment (Table 4). The three *Rhizobium* spp. were evaluated at each storage period, and the peat from Peru was excluded from the statistical analysis (Table 5). Viable counts differed significantly ($P < 0.01$) for the three *Rhizobium* spp., peat samples, and the method of peat sterilization at each storage period. These differences were consistent throughout the shelf life of the inoculants. Significant interactions among the *Rhizobium* spp. (*R*), peat (*P*), and the method of peat sterilization (*S*) were evident at different storage periods. Of these interactions, only $R \times P \times S$ persisted throughout the shelf life of the inoculants. The $R \times S$ interaction was significant only at 24 weeks, and the $R \times P$ interaction was evident in the 4- and 24-week-old inoculants. An analysis of variance ($\alpha = 0.05$ and Duncan's multiple range test) to compare the differences between inoculants produced with irradiated and autoclaved peat samples indicated that the quality of inoculants (viable number of rhizobia per gram of inoculant) in irradiated peat

TABLE 5. Analysis of variance on the compatability of diluted cultures of three *Rhizobium* spp. for inoculant production with diverse peat samples[a]

Source of variation	df	Mean square variation in viable counts after storage for (weeks)[b]:		
		2	4	24
Rhizobium (*R*)	2	0.440**	0.845**	11.953**
Peat (*P*)	3	0.321**	0.618**	0.847**
$R \times P$	6	0.060	0.128**	0.224**
Carrier sterilization (*S*)	1	0.929**	1.511**	0.176*
$R \times S$	2	0.017	0.030	0.102*
$P \times S$	3	0.487**	0.344**	0.051
$R \times P \times S$	6	0.055**	0.045*	0.133**
Error	48	0.034	0.013	0.020

[a] Peat from Peru was not included in the analysis.
[b] *,**, Significance at the 0.01 and 0.001 levels of probability, respectively.

TABLE 6. Quality of inoculants produced with presterilized peat and liquid cultures of rhizobia[a]

Method of inoculant preparation	Log_{10} (no. of rhizobia/g of inoculant) after storage for (weeks):		
	2	4	24
Diluted culture and irradiated peat	9.36 (a)[b]	9.43 (a)	8.73 (a)
Diluted culture and autoclaved peat	9.31 (a)	9.14 (b)	8.63 (b)

[a] Information is a combined analysis of the same raw data obtained for compatibility experiment (Table 4).
[b] Means in the same column with the same letter are not significantly different by Duncan's multiple range test ($\alpha = 0.05$).

TABLE 7. Quality and shelf life of peat inoculants prepared by the tray method compared with the aseptic method

Rhizobium sp.	Log_{10} (no. of rhizobia/ml of culture):		Log_{10} (no. of rhizobia/g of moist peat)[a] after storage for (weeks):										
			2				12			24			
			Autoclaved[c]			Irradiated[d]	Autoclaved		Irradiated	Autoclaved			Irradiated
	Undiluted[b]	Diluted[b]	CR, BG[e]	MPN[f]	MFIF[g]	CR, BG	CR, BG	MPN	CR, BG	CR, BG	MPN		CR, BG
R. phaseoli TAL 182	8.84	6.24	9.38, 9.56	9.26[h]	ND[i]	9.26, ND	9.11, 8.68	8.92	7.67, 8.03	8.55, 8.55	8.08		8.24, 8.26
R. meliloti TAL 380	8.91	6.67	9.65, 9.73	9.76[h]	9.41	9.75, ND	9.41, 9.22	8.84	8.48, 8.35	9.29, 9.17	9.39		9.15, 9.08
Rhizobium sp. TAL 169	9.13	6.81	9.33, 9.26	9.36[h]	9.23	9.80, 9.76	9.41, 8.93	9.69	9.41, 9.33	9.23, 9.08	7.42		9.08, 9.01
Rhizobium sp. TAL 651	9.10	6.95	9.25, 9.56	9.66[h]	9.00	9.75, 9.63	9.20, 9.26	9.45	9.39, 9.48	9.22, 9.16	9.05		9.02, 9.02
Rhizobium sp. TAL 82	9.18	6.70	9.68, 9.69	9.25[j]	9.08	9.33, 9.30	9.51, 9.39	8.52	7.79, 7.87	9.58, 9.12	9.00		7.88, 7.76
R. japonicum TAL 379	9.22	7.12	9.62, 7.21	9.25[j]	9.38	9.72, 8.76	9.64, 9.44	9.18	9.60, 9.42	9.38, ND	9.05		9.00, ND

[a] Counts are means of three replications.
[b] Number of viable rhizobia in the liquid culture before incorporation into irradiated and autoclaved peat, respectively.
[c] Counts of inoculants prepared by the tray method with autoclaved peat.
[d] Counts of inoculants prepared by injecting undiluted culture into gamma-irradiated peat.
[e] CR, BG, Plate counts on CRYMA and BGYMA media, respectively.
[f] MPN counts by plant infection.
[g] MFIF counts.
[h] MPN counts in plastic growth pouches.
[i] ND, Not done.
[j] MPN counts in enclosed seedling-agar tubes.

was significantly higher than in the autoclaved peat for all the storage periods (Table 6).

In the combined analysis of the raw data from the factorial experiment (Table 4), the order of strain performance was *R. japonicum* TAL 102 > *Rhizobium* sp. strain TAL 82 > *R. phaseoli* TAL 182, regardless of peat source or method of peat sterilization. The mean viable counts per gram of inoculant, in order of strain performance, were log 9.23, log 8.92, and log 7.89 after 24 weeks of storage at 28°C.

The same analysis revealed that the peat from Argentina was the most superior for the three *Rhizobium* spp., with a mean viable count of log 9.0 per g of inoculant at the end of the storage period. The mean counts in the other peat samples were log 8.63 (United States), log 8.56 (Mexico), and log 8.54 (Spain).

Inoculant preparation by the tray method. The quality and shelf life of the inoculants produced by the tray method with six *Rhizobium* spp. are summarized in Table 7. Enumeration of the rhizobia in these inoculants was done by the spread plate, MPN, and MFIF (done only on the 2-week-old inoculants) methods. The counts at 2 weeks indicated that the diluted cultures multiplied in the autoclaved peat in the presence of contaminants which were introduced during the septic mixing operation. The inoculants were still of acceptable quality at 12 and 24 weeks, although higher counts were observed at 12 weeks.

Even though the inoculants prepared by the tray method were packaged immediately after mixing, the contaminant increase was considerable, averaging from 35% (at 2 and 12 weeks) to 49% (at 24 weeks) of the CFU. The CFU clearly distinguishable as those of the *Rhizobium* inoculant strain on the basis of colony appearance on the plating medium were further examined by immunofluorescence. Results of the immunofluorescence examination were generally positive with few negative reactions, but the number of examinations (total of 15 CFU) for each replicated treatment was insufficient for quantitative analysis. An earlier experiment with the tray method was abandoned because of very serious contamination which resulted from not wearing sanitized polyethylene disposable gloves during mixing. Scrupulous cleaning of the hands before mixing in of the inocula was inferior to sanitization provided by the polyethylene gloves.

Since plate and MPN counting techniques were routinely used for the tray method to evaluate multiplication of the rhizobia from diluted cultures, the reliability of these techniques was examined. The data in Table 7 were analyzed for correlation between plate counts (i.e., CRYMA versus BGYMA) and between plate counts and MPN (i.e., CRYMA versus MPN and BGYMA versus MPN). Results of both the tray and aseptic methods were analyzed. The results were (i) tray method, CRYMA versus BGYMA, $r = 0.73$ ($P < 0.001$); CRYMA versus MPN, $r = 0.44$ (not significant); BGYMA versus MPN, $r = 0.20$ (not significant); and (ii) aseptic method, CRYMA versus BGYMA, $r = 0.98$ ($P < 0.001$). These determinations were especially important in accepting the data from plate counts which involved selective recognition of colonies of *Rhizobium* spp. in the presence of contaminants, particularly for the tray method. The spot checks on single colonies by immunofluorescence further supported the acceptability of the plate counts. The MPN counts were not significantly correlated with the plate counts on CRYMA or BGYMA. In the analysis, results of *R. japonicum* TAL 379 were not included because brilliant green inhibited the growth of this strain, especially in the young peat inoculants. The counts by MFIF were somewhat lower than the counts obtained by other methods and showed no significant relationship with those from the other methods.

Low-temperature (4°C) storage of inoculants produced by the tray method. Inoculants prepared by the tray method were aged to 3 weeks and transferred to storage at 4°C for 23 weeks. A parallel experiment with the same *Rhizobium* spp. in irradiated peat was set up. The data (Table 8) were analyzed by a paired *t* test, and the results indicated a

TABLE 8. Influence of low storage temperature (4°C) and time (23 weeks) on the survival of rhizobia in inoculants prepared by the tray and aseptic methods

Rhizobium spp.	Log_{10} (no. of rhizobia/g of peat)[a]				
	Autoclaved (tray method)			Irradiated (aseptic method)	
	MPN[b]	Initial[c]	Final[d]	Initial	Final
R. phaseoli TAL 182	9.01	9.49, 9.31[e]	9.19, 9.20	8.27, 8.24	6.94, 6.59
R. meliloti TAL 380	9.85	9.97, 9.89	9.76, 9.71	9.42, 9.36	9.35, 9.32
Rhizobium sp. TAL 169	9.58	9.44, 8.94	9.35, 8.96	10.00, 9.97	9.07, 9.05
Rhizobium sp. TAL 651	9.61	9.38, 9.41	8.59, 8.47	9.49, 9.61	9.33, 9.40
Rhizobium sp. TAL 82	9.25	9.73, 9.64	9.47, 9.46	9.42, 9.43	6.72, 6.82
R. japonicum TAL 379	9.03	9.88, 8.68	9.63, ND	9.98, 9.51	9.62, ND
Means		9.55	9.25	9.38	8.38

[a] Counts are mean of three replicatons.
[b] Viable counts of the 3-week-old inoculants (tray method only) by the plant infection (MPN) technique before storage at 4°C.
[c] Initial counts were taken after 3 weeks of storage at 28°C after which inoculants were immediately transferred to storage at 4°C for 23 weeks. Liquid cultures were diluted for the tray method but not for the aseptic method with the irradiated peat.
[d] Plate counts after 23 weeks of storage at 4°C.
[e] Plate counts on CRYMA and BGYMA, respectively.

significant ($P < 0.001$) overall decrease in the viability of the rhizobia in the inoculants prepared by both the methods. The data were also analyzed for correlation between plate counts used in both methods. The results for the tray method were as follows: initial counts, CRYMA versus BGYMA, $r = 0.84$ ($P < 0.05$); final counts, CRYMA versus BGYMA, $r = 0.89$ ($P < 0.05$). With the aseptic method the results were as follows: initial counts, CRYMA versus BGYMA, $r = 0.94$ ($P < 0.05$); final counts, CRYMA versus BGYMA, $r = 0.99$ ($P < 0.01$).

Inoculant effectiveness. Inoculants of *R. japonicum* TAL 102, *R. phaseoli* TAL 182, and *Rhizobium* sp. strain TAL 82 prepared with diluted cultures incorporated in autoclaved and irradiated peat from the various countries were plant tested. The results (Table 9) showed that the effectiveness of the inoculants prepared with diluted cultures were not significantly different from those of the multistrain inoculants from NifTAL as measured by plant shoot dry weight. Examination of nodule sections showed pink to red pigmentation, indicative of effective symbiosis.

TABLE 9. Plant inoculation tests for effectiveness of inoculants prepared with diluted cultures of *Rhizobium* spp. and peat from different countries

Inoculation treatments and controls	Peat presteril-ization[b]	Dry wt of shoot (g)[a] with:		
		R. japonicum (TAL 102)	*R. phaseoli* (TAL 182)	*Rhizobium* sp. (TAL 82)
Mexico	A	3.37	2.75	0.73
	G	3.48	2.42	0.81
Argentina	A	3.41	2.90	0.80
	G	3.34	3.40	0.91
Spain	A	3.44	3.00	0.85
	G	3.53	2.37	0.79
United States (Demilco)	A	2.42	3.23	0.67
	G	2.73	3.68	0.78
Peru	A	2.44	ND[c]	ND
	G	2.30	ND	ND
NifTAL inoculant[d]	G	2.74	2.81	0.88
Control (uninoculated)		0.83	0.98	0.16
Control (70 ppm of N)		4.18	6.00	1.79

[a] Mean of three replications with two plants per replication; controls were not included in the statistical analysis.
[b] A, Autoclaving; G, gamma irradiation.
[c] ND, Not done.
[d] NifTAL multistrain inoculant prepared with undiluted cultures of *Rhizobium* spp.

DISCUSSION

The potential for using diluted cultures of *Rhizobium* spp. with autoclaved peat for inoculant production is demonstrated in this investigation. The significance of using diluted cultures lies in the fact that the *Rhizobium* spp. must multiply and survive in high numbers in the presterilized peat on introduction. On reaching maximum numbers, the rhizobia should reflect characteristics parallel or similar to those of strains in peat inoculants prepared in the normal procedure whereby undiluted cultures and presterilized peat are used.

The use of water as a suitable diluent without detrimental effects, as evidenced in all the experiments described in this work, has two interesting implications.

First, water brings out the intrinsic attributes of peat, indicating that the peat samples used for inoculant production are naturally self sufficient in the nutrients required for multiplication and long-term survival of *Rhizobium* spp. in the inoculants. However, the carry-over of unmetabolized YM nutrients may not be ignored during initial stages of multiplication, but long-term influence of nutrient carry-over is probably negligible. The exact mechanisms of nutrient extraction by the rhizobia from the peat during growth and survival (especially during long-term storage) have not been established, despite widespread speculations about nutrient release from the peat during sterilization. The chemical nature, array, and concentration of the constituent nutrients and the influence of sterilization on their release and availability for growth and survival of *Rhizobium* spp. warrants further research. It is conceivable that the peat environment is more complex than is reflected by its basic chemical composition (6). It was shown that undiluted and 1,000-fold-diluted fully grown cultures of *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 showed no differences in the survival or shelf life of the inoculants in irradiated peat (16).

Second, the suitability of water as the diluent may have

economic implications for inoculant production systems with presterilized peat, in that sterilized water can be used for diluting smaller volumes of fully grown cultures of *Rhizobium* spp. before incorporation into autoclaved or irradiated peat. Furthermore, diluting liquid cultures significantly reduces the size and capacity of expensive fermenters and accessories needed for growing rhizobia without loss in the production capacity of inoculant production plants. A prototype system for producing inoculants with diluted cultures and presterilized peat has been validated and will be published elsewhere. The use of diluted cultures and presterilized peat (autoclaved in polypropylene bags) may be a cheaper option for small- to medium-scale inoculant production compared with the use of undiluted cultures and gamma-irradiated peat.

The wider applicability of the dilution technique is further supported by its performance with peat from Mexico, Argentina, Spain, the United States, and Peru (Tables 4 and 6). The slight differences in quality between inoculants produced with autoclaved and irradiated peat, although statistically significant, should have no practical drawbacks, as these inoculants had 10^9 rhizobia per g at manufacture and 10^8 rhizobia per g after 6 months of storage, thus meeting quality requirements set for sterilized peat (13). This slightly lower quality in inoculants produced with diluted cultures is not related to the dilution process, as the difference has been shown to occur with autoclaved peat and undiluted cultures (14). Inoculants produced with water-diluted cultures and presterilized peat (autoclaved or irradiated) did not suffer from any lack of effectiveness of the rhizobia or indicate the restriction of the method to any one peat or *Rhizobium* strain (Table 9). Three *R. japonicum* strains and two slow-growing cowpea-type *Rhizobium* strains were found to remain viable and able to rapidly nodulate their respective hosts after being stored in purified water at ambient temperature for a period of at least 1 year (5).

Differences in multiplication and survival in autoclaved peat (Table 2) were noted between *R. japonicum* TAL 102 and *R. phaseoli* TAL 182. Maximum populations of *R. japonicum* TAL 102 were not attained until 2 weeks longer than those of *R. phaseoli* TAL 182. A similiar lag phase in the multiplication was shown by *Rhizobium* sp. strain TAL 169 (Table 3). Such lag phases in multiplication in autoclaved peat may be considered undesirable traits for potential inoculant strains from the viewpoint of inoculant readiness for distribution and incompatibility with autoclaved peat. The reasons for the slower multiplication in autoclaved peat by some rhizobia have not been established. These examples of sensitivity of rhizobia to multiplication in autoclaved peat may not be widespread, as high-quality inoculants of several *Rhizobium* spp. were produced with autoclaved peat (19, 21).

Viability of the rhizobia during storage at 28 or 4°C after maturation gave mixed results, indicating that there was a strain preference for storage temperatures. Long-term storage at 28°C usually results in a decline in the population, and this was observed with all *Rhizobium* spp. in the peat samples from the various countries. The decline in population at 28°C was not related to use of diluted cultures, as similar observations were made on rhizobia in peat inoculants prepared with irradiated peat and undiluted cultures (17). With peat after maturation at 28 to 30°C, low-temperature (4°C) storage is generally more favorable for survival than storage at higher temperatures, including those at which growth would normally occur, e.g., 26°C (20).

Also, Thompson (20) reported that storage at 4°C was favorable to fast-growing *Rhizobium* strains, and survival of slow-growing strains was superior at 26°C. Survival of slow-growing strains such as CB 82 (for fine stem stylo), CB 627 (for *Desmodium* spp.), and CB 1024 (for *Lablab purpureus*) was better at 28°C than at 4°C (12).

In this investigation, fast-growing strains of *R. phaseoli* TAL 182 and TAL 1376 and *Rhizobium* sp. strain TAL 82 from *Leucaena leucocephala* indicated significant loss in viability when stored at 4°C. Similarly, inoculants of slow-growing *Rhizobium* sp. strain TAL 651 did not indicate good storage characteristics at 4°C. Low-temperature storage was not always detrimental, as two other strains *Rhizobium* sp. strains TAL 169 and TAL 620 showed significant increases in the population.

The experiment on the compatibility of the dilution approach for inoculant production with various peats and strains indicated significant survival differences among *Rhizobium* strains which could be related to the peat, method of sterilization of the peat, and various interactions (Table 5).

Strain survival differences with various peats has been well established (14). Marked *Rhizobium*-carrier interactions were demonstrated with *R. meliloti*, *R. japonicum*, and a cowpea *Rhizobium* sp. (18). Sterilization of peat by autoclaving was inferior to gamma irradiation with three *Rhizobium* spp. (14). Strains of *R. japonicum* and *R. meliloti* had significantly higher population in peat presterilized by gamma irradiation at 50 than at 25 kGr (19).

These data clearly demonstrate that the survival of *Rhizobium* spp. in peat is influenced by various factors which are common in inoculant production. As long as strain differences and interactions do not severely limit multiplication and survival, leading to low-quality inoculants, any new method of inoculant production should be considered feasible. Such a method is described in this investigation.

The general inference of the tray method (Table 7) of inoculant production was that rhizobia in a water-diluted culture (e.g., 10^6 to 10^7 cells ml^{-1}) were able to multiply to high populations in autoclaved peat in the presence of low numbers of aerial microorganisms introduced at the time of mixing with the peat. Once the high populations were attained, these populations survived like rhizobia in inoculants produced by the normal procedure. The critical factors thay may have influenced initial multiplication of *Rhizobium* spp. were probably the brief period of peat sterility, low numbers of introduced aerial microorganisms relative to the high numbers of *Rhizobium* spp. present in the diluted culture, the use of sanitized polyethylene gloves during mixing, and the immediate packaging of the inoculants. Posttreatment contaminants in gamma-irradiated peat can occasionally reach levels of 10^7 per g, and even at this level the survival of rhizobia is not seriously affected (10). Survival of *R. japonicum* WB1 in irradiated peat was high (log 9.254 per g of inoculant) after 6 months of storage at 27°C in the presence of contaminants (19). High-quality inoculants can be produced with partially sterilized peat (4). Keeping contamination to the lowest possible levels is the best option for producing good-quality inoculants by the tray method, and this is emphasized as a important prerequisite. Specially constructed mixing cabinets which can be easily fumigated or sterilized may be better than the regular laboratory environment for the tray method of inoculant production.

The close relationship (as measured by the correlation coefficient, *r*) of the plate count data suggested that recognition of *Rhizobium* colonies in the presence of contaminants was not a severe problem in the enumeration of inoculants

produced by the tray method, even though inclusion of contaminants in the counts could not be entirely eliminated. The lower r values for the tray method in comparison with that for the aseptic method indicated that the enumeration may have been subject to errors introduced by inclusion of contaminants. The lack of correlation between the standard MPN (plant infection) counts as described by Vincent (23) gave significantly lower estimates of *Rhizobium* numbers in soil (3), and a modification of the standard MPN produced higher and more definitive population estimates (1). However, despite its inaccuracy, the standard MPN method was useful in estimating the *Rhizobium* population relative to other methods used in the validation of the tray method for inoculant production in this investigation. The MFIF technique used in this investigation gave comparable results in relation to the other techniques, but it must be developed further for its application in inoculant quality control.

ACKNOWLEDGMENTS

This work was supported by the U.S. Agency for International Development, contract no. ta-C-1207 (NifTAL Project, University of Hawaii).

The skilled technical assistance of J. Pinchin and B. Martin is gratefully acknowledged. I also thank F. Cady for statistical analysis of some of the data.

LITERATURE CITED

1. **Biederbeck, V. D., and B. D. Waker.** 1981. PEPI: an improved plant infection technique for enumeration of rhizobia, p. 536–561. *In* K. W. Clark and J. H. G. Stephens (ed.), Proceedings, 8th North American *Rhizobium* Conference. University of Manitoba, Winnipeg, Canada.
2. **Bohlool, B. B., and E. L. Schmidt.** 1968. Nonspecific staining: its control in immunofluorescence examination of soil. Science **162:**1012–1014.
3. **Boonkerd, N., and R. W. Weaver.** 1982. Cowpea rhizobia: comparison of plant infection and plate counts. Soil Biol. Biochem. **14:**305–307.
4. **Burton, J. C.** 1967. *Rhizobium* culture and use, p. 1–33. *In* H. J. Peppler (ed.), Microbial technology. Van Nostrand Reinhold, New York.
5. **Crist, D. K., R. E. Wyza, K. K. Mills, W. D. Bauer, and W. R. Evans.** 1984. Preservation of *Rhizobium* viability and symbiotic infectivity by suspension in water. Appl. Environ. Microbiol. **47:**895–900.
6. **Date, R. A., and R. J. Roughley.** 1977. Preparation of legume seed inoculants, p. 243–275. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation. Section IV: agronomy and ecology. John Wiley & Sons, Inc., New York.
7. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
8. **Hoben, H. J., and P. Somasegaran.** 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44:**1246–1247.
9. **Newbould, F. H. S.** 1951. Studies on human type legume inoculants. I. Growth and survival in storage. Sci. Agric. **31:**259–265.
10. **Parker, F. E., and J. M. Vincent.** 1981. Sterilization of peat by gamma-irradiation. Plant Soil **61:**285–293.
11. **Persaud, H. B.** 1977. Problems in developing an inoculant capability, p. 441–496. *In* J. M.Vincent, A. S. Whitney, and J. Bose (ed.), Exploiting the legume-*Rhizobium* symbioses in tropical agriculture. Misc. publ. no. 145. University of Hawaii, College of Tropical Agriculture, Honolulu.
12. **Roughley, R. J.** 1982. The storage, quality control and use of legume seed inoculants, p. 115–126. *In* P. H. Graham and S. C. Harris (ed.), Biological nitrogen fixation technology for tropical agriculture. Centro Internacional de Agricultura Workshop. CIAT plublication no. O3E-5(82). Centro Internacional de Agricultura Tropical, Cali, Colombia.
13. **Roughley, R. J., and D. J. Pulsford.** 1982. Production and control of legume inoculants, p. 192–209. *In* J. M. Vincent (ed.), Nitrogen fixation in legumes. Academic Press, Sydney.
14. **Roughley, R. J., and J. M. Vincent.** 1967. Growth and survival of *Rhizobium* spp. in peat culture. J. Appl. Bacteriol. **95:**362–376.
15. **Schmidt, E. L., R. D. Bankole, and B. B. Bahlool.** 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. **95:**1987–1992.
16. **Somasegaran, P., and J. Halliday.** 1982. Dilution of liquid *Rhizobium* cultures to increase production capacity of inoculant plants. Appl. Environ. Microbiol. **44:**330–333.
17. **Somasegaran, P., V. G. Reyes, and H. J. Hoben.** 1984. The influence of high temperatures on the growth and survival of *Rhizobium* spp. during preparation, storage and distribution. Can. J. Microbiol. **29:**23–30.
18. **Strijdom, B. W., and C. C. Deschodt.** 1976. Carriers of rhizobia and the effects of prior treatment on the survival of rhizobia. Int. Biol. Programme **7:**151–168.
19. **Strijdom, B. W., and H. J. van Rensburg.** 1981. Effect of steam sterilization and gamma irradiation of peat on quality of *Rhizobium* inoculants. Appl. Environ. Microbiol. **41:**1344–1347.
20. **Thompson, J. A.** 1980. Production and quality control of legume inoculants, p. 489–533. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Inc., New York.
21. **van Schrevan, D. A.** 1958. Methods used in the Netherlands for the production of legume inoculants, p. 328–333. *In* E. G. Hallsworth (ed.), Nutrition of the legumes. Butterworth and Co., London.
22. **van Schrevan, D. A.** 1970. Some factors affecting growth and survival of *Rhizobium* spp. in soil-peat cultures. Plant Soil **32:**113–130.
23. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. Int. Biol. Programme **15:**54–58.
24. **Weaver, R. W., and L. R. Frederick.** 1972. A new technique for most-probable-number counts of rhizobia. Plant Soil **36:** 219–222.

ILLUSTRATED CONCEPTS IN AGRICULTURAL BIOTECHNOLOGY

A series from the NifTAL Project-MIRCEN, Department of Agronomy and Soil Science*
College of Tropical Agriculture and Human Resources
University of Hawaii

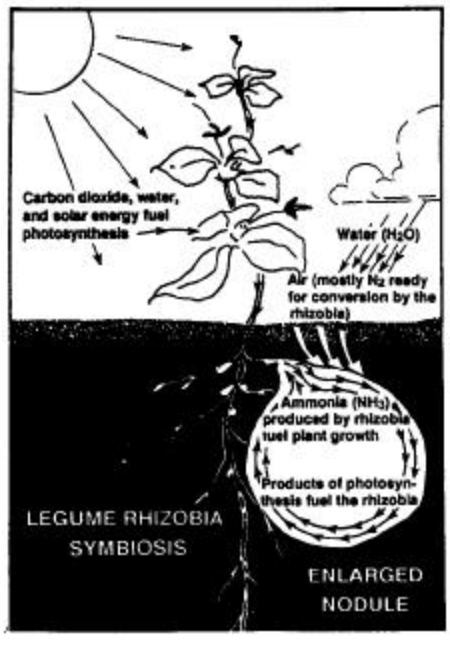
Biological Nitrogen Fixation (BNF): Commonly asked Questions and Answers

About nitrogen

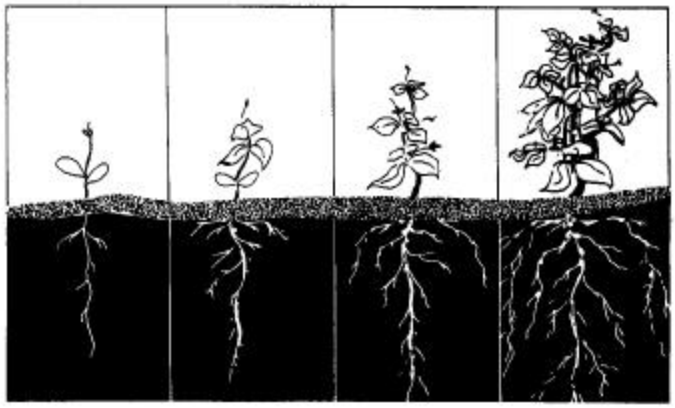
All living things require nitrogen to make proteins needed for life. Although nitrogen gas makes up about 80% of the air we breathe, most living things cannot use atmospheric nitrogen and require that it be combined or "fixed" with other elements like oxygen and hydrogen before it can be assimilated.

Animals get the nitrogen they need by consuming plant or animal protein, while most plants get fixed nitrogen from the soil. Because soils are often low in nitrogen content, good plant growth often means supplementing soil nitrogen with fertilizer nitrogen which is expensive to produce and is, therefore, too costly for many small farmers to buy. Fortunately, some plants can form mutually beneficial relationships (symbioses) with microorganisms which convert atmospheric nitrogen to ammonia. This "fixed" form of nitrogen is then used by the plants to make proteins. The name of this conversion process is biological nitrogen fixation or BNF.

How BNF Works



When the right rhizobia are present, legumes can fix nitrogen throughout their entire life cycle. Healthy plants and high seed yields are the result.



Can any plant form a symbiosis with a nitrogen fixing microorganism?

No. Only certain types of microorganisms and certain plants can form nitrogen fixing symbioses. For example, many species of the legume family form nitrogen fixing symbioses with common soil bacteria called rhizobia. This ability partly accounts for the high protein content of legume seeds--the main protein source for most of humankind. Cereals, the other major component of human diets cannot form nitrogen fixing symbioses. Many of the plant species that form nitrogen fixing symbioses are important in agriculture and forestry.

Can a plant get all the nitrogen it needs from biological nitrogen fixation?

Yes, in theory, but even nitrogen fixing plants will always take up some nitrogen from the soil. This is an important point to remember when intercropping nitrogen-fixing and non-nitrogen- fixing crops is being recommended to farmers. Although the nitrogen-fixing plant can produce some or most of its nitrogen, it can also compete with the non-fixing crop for soil or fertilizer nitrogen.

Do legumes that can fix nitrogen always do so?

No. In the case of a nitrogen-fixing legume, there must first be rhizobia bacteria and a susceptible legume plant present. In addition, appreciable quantities of nitrogen are fixed only by healthy plants. Therefore, other plant nutrients (for example, phosphorus and potassium), as well as the right environmental conditions (like soil moisture temperature, and salinity), must be optimum for good plant growth. Nitrogen fixation can only supply nitrogen to the plant, it cannot make up for other nutritional or environmental deficiencies.

If rhizobia are common soil bacteria, don't they occur everywhere?

No. There are many different types of rhizobia and not all types occur in all soils. This is important because certain plant species, like soybeans, will only form a nitrogen fixing symbiosis with certain rhizobia which may not be present in the soil in which the legume is to be planted. This situation occurs most often when a new crop requiring a specific type of rhizobia is planted in a field for the first time. Finally, though certain legumes such as cowpea for ex-

ample, can form nitrogen fixing symbioses with several types of rhizobia, they usually fix the most nitrogen with only one type.

How does one know whether the "right" rhizobia for a particular crop is in a field?

If ample numbers of nodules form naturally on a legume crop, one may conclude that the field contains native rhizobia that can nodulate that crop. If, upon cutting open the nodules, the inside is pink or red, one may also assume that the "native" rhizobia have formed an active nitrogen fixing symbiosis with that crop species. However, neither of these simple tests will tell whether the native rhizobia are the "best" (fix the most nitrogen) on that crop.

How can one insure that the "best/right" rhizobia establishes a nitrogen fixing symbiosis with a legume?

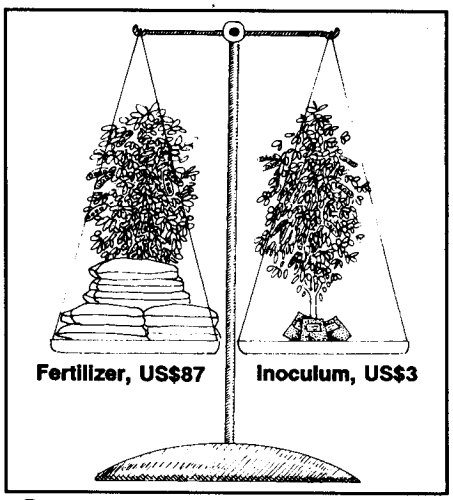
One can insure the maximum benefits from nitrogen fixation by inoculating the seeds with the "best" rhizobia. This insures that when the root emerges from the seed it is in immediate contact with the "right/best" rhizobia.

What is inoculation and how is it done?

Inoculation is the simple process of coating seeds with inoculant prior to planting. In the case of legumes, inoculant is a mixture of rhizobia and an inert material such as powdered peat. Alternatively, the inoculant can be placed in the furrows in which the seeds will be planted or banded alongside the seeds after planting. For additional information on when to use the different methods and for detailed instructions on how to inoculate, contact the NifTAL Project.

Is inoculant expensive?

Rhizobial inoculant is relatively inexpensive (see diagram below). It would take at least U.S.$87 worth of urea to produce a soybean yield comparable to that possible using only U.S.$3 worth of inoculant.



Cost comparison of inoculant vs. fertilizer nitrogen

How does one know if a legume crop is getting enough nitrogen from nitrogen fixation?

The simplest way is to plant one part of a field with inoculated seeds, one part with uninoculated seeds, and a third part with uninoculated seeds but then adding nitrogen fertilizer. By comparing the yields in the three parts, one can get an idea of whether nitrogen fixation is meeting most of the nitrogen demand of the crop.

However, remember that getting the most out of biological nitrogen fixation, whether for a grain or tree legume, requires that the best rhizobia for a particular plant species is either in the field or supplied to the plant via inoculation and that all other management conditions are optimal.

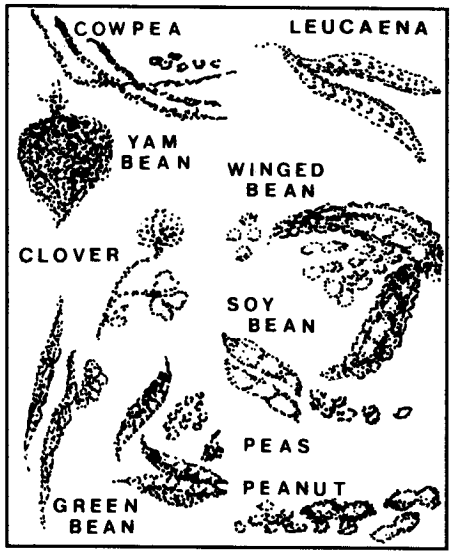
Where can inoculant be obtained?

Rhizobial inoculants for many crop legumes are manufactured in a number of countries around the world. The local agricultural extension agency or national departments of agriculture are the best places to seek information on whether inoculants are locally manufactured or have to be imported. Inoculants for tree legumes are not yet being commercially produced and so must be obtained from sources like NifTAL or other research or development agencies.

If there are no local sources of rhizobial inoculant where can it be obtained?

Contact NifTAL whose mandate is to supply small quantities of inoculant to all interested individuals for test purposes. Should sufficient local interest be generated, NifTAL can subsequently help national or regional institutions establish a BNF research program and/or examine the feasibility of establishing an in-country inoculant production capability.

Some Useful Legumes



By Joann P. Roskoski

*The NifTAL Project (1000 Holomua Avenue, Paia, Maui, Hawaii 96779-0779 USA) an agricultural research and development project funded by USAID. Telephone: 808 579 9568/Fax 808 579 8516; Cable: NifTAL; Bitnet: NifTAL@UHCCUX; CGNET: CGI 056

ILLUSTRATED CONCEPTS IN AGRICULTURAL BIOTECHNOLOGY

A series from the NifTAL Project*-MIRCEN, Department of Agronomy and Soil Science
College of Tropical Agriculture and Human Resources
University of Hawaii

A SMALL GLASS FERMENTER FOR THE PRODUCTION OF RHIZOBIUM INOCULUM

Legumes can obtain a substantial amount of nitrogen for their growth and nutrition in symbiosis with effective N-fixing rhizobia in their root nodules. Rhizobial inoculants for seed or soil inoculation may be prepared (Figure 1) by blending fermenter cultured rhizobia with suitable carriers such as finely ground peat. A glass fermenter has been devised which may be used for the production of sufficient volumes of broth cultures to meet the requirements of large field experiments on *Rhizobium* inoculation. In addition, this glass fermenter is also suitable for the production of starter cultures for medium sized commercial fermenters.

Materials

All materials required are readily available. The items needed are: One 4 liter Erlenmeyer flask to which a 35 mm long glass tube (7 mm OD) has been fitted near its base and parallel to the bottom; glass tubing, 800 mm x 7 mm OD (cut into 6 pieces: 1 @ 400 mm, 5 @ 80 mm); two pieces of larger diameter glass tubing, 120 mm x 30 mm OD (the barrels of two disposable 50 ml plastic polypropylene syringes may be used instead); a piece of solid glass rod, 3 cm x 8 mm OD; surgical rubber tubing, 800 mm x 12 mm OD (6 mm ID); four #6.5 rubber stoppers; one #10 rubber stopper; small roll aluminum foil; small roll of non-absorbent cotton wool; tubing clamp; 300 mm flexible steel wire; autoclavable masking tape; small air pump such as an aquarium pump; YMB broth (mannitol - 10 g; yeast extract - 0.5 g; $MgSO_4 \cdot 7H_2O$ - 0.2 g; K_2HPO_4 - 0.5 g; NaCL -0.1 g. Dissolve in 1 liter of distilled H_2O, adjust pH to 6.8 and autoclave.)

Assembly

The assembly is shown in Figure 2 (diagram) and Figure 3 (photograph). It is important to pack the cotton in the filter barrels uniformly but loosely enough to facilitate air passage. All glass tubes and stoppers have to be tightly fitted to avoid leaks. Support for the filters is achieved by a wire ring surrounding the lower neck of the vessel. Two small pieces of wire are bent into little hooks which are taped to the ends of the filters. The filters may then be hooked into the wire ring.

Operation

A maximum of three liters of yeast mannitol broth may be filled into the vessel. The mouth of the flask is then tightly stoppered and secured with a wrapper of cotton wool followed by aluminum foil. The air inflow is closed with a clamp near that point where the inflow filter connects to the 400 mm inlet tubing. The unit is then autoclaved at 15 lbs. and 121°C for 1 hour. Inoculation is done on a clean bench after the broth has cooled to room temperature. Twenty (20) ml of inoculant broth from a shaker culture is aseptically injected through the latex inlet tubing with a sterilized syringe fitted with an 18 g needle. An aquarium pump is then attached to the inflow tubing, the clamp removed, and the airflow activated. A fully grown culture of 1×10^9 cells/ml can be expected after 4 days for fast growing rhizobia and 6 days for most slow growers.

Production Potential

For irradiated peat, approximately 40 ml (a trial injection of broth to peat ratio must be done to insure correct moisture content) of fermenter culture is aseptically injected into 50 g peat to give 90 g of inoculant. This results in 75 bags or 6.75 kg of moist inoculant from 3 liters of broth culture. Commercial inoculation rates for large seeded legumes, e.g., soybean, is around 0.3 kg inoculant per 65 kg seed (good for planting 1 hectare) which provides approximately 2.5×10^5 rhizobia per seed. Using these figures as guidelines, 6.75 kg of inoculant prepared from 3 liter of fermenter culture can be used to inoculate 1527.5 kg soybean seeds for planting 23.5 hectares.

For inoculating small-seeded species, e.g., alfalfa, the commercial rate is 70 g per 16 kg seed (good for planting 1 hectare) to provide approximately 5×10^3 rhizobia per seed. In this case, the 6.75 kg of alfalfa inoculant from 3 liters of culture will be sufficient to inoculate 1542.4 kg of alfalfa seed for planting 96.4 hectares.

Comparative Economic Value

The net economic gain per inoculated crop was estimated by using U.S. commercial production yields and a cost of US$.20 per Kg of Urea fertilizer. Thus, for an equivalent benefit from nitrogen, the per hectare cost of urea fertilizer would be US$435 for alfalfa (2176 Kg per hectare) and US$87 for soybean (435 Kg per hectare).

By Heinz J. Hoben and Padma Somasegaran

*The NifTAL Project (1000 Holomua Road, Paia, Maui, HI 96779-9744) is an agricultural research project funded by USAID.

H. Hoben

Figure 1. Inoculant preparation with broth culture from fermenter.

R. Gabrielson

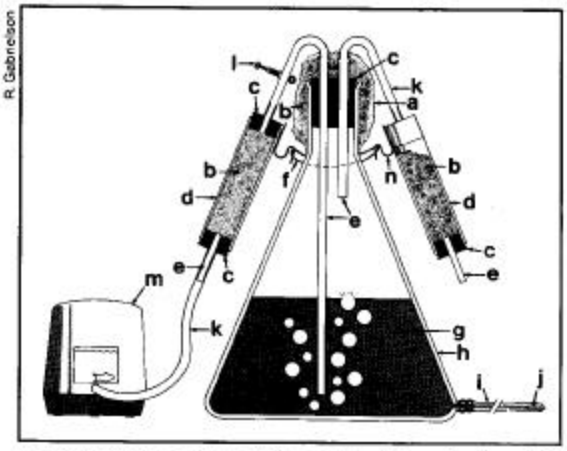


Figure 2. Scheme of simple fermenter unit. a. aluminum foil outer wrap; b. nonabsorbent cotton wool; c. autoclavable stoppers; d. glass or plastic syringe filter housing; e. glass tubing; f. wire ring; g. growth medium; h. Erlenmeyer flask; i. sampling tubing; j. glass plug; k. latex tubing; l. hose clamp; m. aquarium pump; n. wire hook.

R. Gabrielson

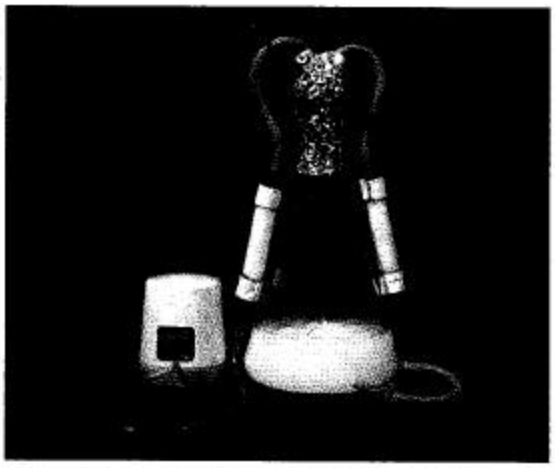


Figure 3. Assembled fermenter containing inoculum.

ILLUSTRATED CONCEPTS IN AGRICULTURAL BIOTECHNOLOGY
A joint publication of the NifTAL Project*/MIRCEN**

A Medium-Scale Fermentor for Mass Culture of Rhizobia

Inoculants for legumes are produced by blending a broth culture of the root nodule bacteria rhizobia with a suitable carrier material such as peat. A major consideration in inoculant production is the mass culture of Rhizobia which is an important factor determining the scale of production. Mass culture requires suitable fermentors. These must be simple enough to allow for easy sterilization, access for inoculation and sampling, aeration and cleaning operations. A low-cost stainless steel fermentor for mass culture of rhizobia for medium-scale commercial inoculant production is described here.

Figure 1. Inoculating the Fermentor

Description.

The complete assembly of the fermentor is shown in Figure 2. The main body of the fermentor is a 141 liter (37.2 gallon) stainless steel pressure vessel modified to NifTAL's specifications. The height:diameter ratio in 2.25:1. It is domed at the top and bottom and held upright by a welded-on stainless steel skirt. The top dome has an oval opening in its center with snap-type closure which uses a special O-ring seal. The following accessories are also located on the top dome: steam pressure gauge (0-30 psi); pressure relief valve (15 psi, factory adjusted); aeration system with in-line filters for the intake and exit of sterile air; inlet and outlet ports for water passage through the built-in stainless steel cooling coil. The inoculation port, thermometer, and the sampling port are positioned in a vertical plane on the wall of the vessel. The fermentor is situated on a sturdy steel support which also houses a 98,000 BTU 4-ring gas burner.

Operation.

The NifTAL fermentor serves initially as an autoclave to sterilize the growth medium and later for cell multiplication upon inoculation. The fermentor has a maximum working capacity of 100 liters. A standard yeast extract-mannitol medium is used for the growth of rhizobia. The boiling point of the growth medium is reached after one hour with the gas burner turned to maximum heat. An additional 45 minutes are required to reach 15 psi at 121 degrees C. This pressure is maintained with low heat by controlling the burner. The medium is sterilized for 45 minutes. While the growth medium sterilization is in progress, the cotton packed pre-sterilized air inlet filter is attached. Following sterilization of the medium, steam released through the air outlet tube will sterilize the glasswool packed outlet filter. Regular tap water run through the cooling coil will drop the temperature to 30 degrees C within one hour.

One liter of starter culture is aseptically introduced through the inoculation port. Aeration is initiated by gradually letting in compressed air (5 psi) through the inlet filter. This sterile air provides aeration as well as agitation for the growth of the rhizobia. Rhizobial growth can be monitored by aseptically removing samples via the sampling port.

Production Potential.

Usually 1 liter of broth culture from the fermentor is blended with 1.5 kg of finely ground peat to give 2.5 kg of peat inoculant. Therefore, 100 liters of culture can be used to prepare 250 kg of inoculant. Commercial inoculation rates for large seeded legumes, e.g., soybeans, are approximately 0.3 kg inoculant per 65 kg seed which provides 2.5×10^5 rhizobia per seed. Using these figures as guidelines, 250 kg of inoculant will for inoculate 54,166 kg of soybean seed (good for planting 833 ha with soybean).

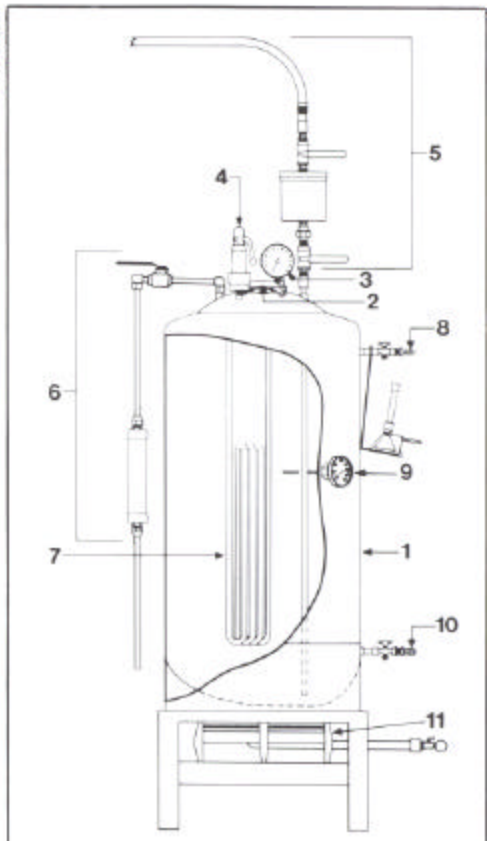


Figure 2. Fermentor Parts

Fermentor vessel(1); snap top closure (2); steam pressure gauge (3); pressure relief valve (4); air inlet assembly (5); air outlet assembly (6); cooling coil (7);inoculation port (8); thermometer (9); sampling port (10); and gas burner (11).

By Padma Somasegaran, Heinz J. Hoben, and Joe C. Burton

*The NifTAL Project (1000 Holomua Avenue, Paia, Maui, HI 96779- 9744 USA) is an agricultural research project funded by USAID and is part of the Dept. of Agronomy and Soil Science, College of Tropical Agriculture and Human Resources, University of Hawaii.

** MIRCEN (Microbiological Resource Center) is a designation of the United Nations Educational, Scientific and Cultural Organization.

(8/1988) Number 3

ILLUSTRATED CONCEPTS IN AGRICULTURAL BIOTECHNOLOGY

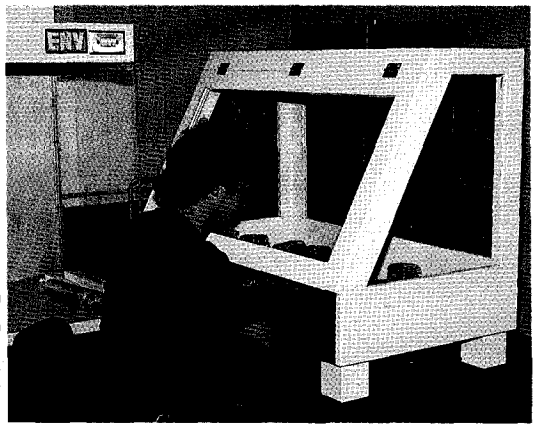
A series from the NifTAL Project-MIRCEN, Department of Agronomy and Soil Science*
College of Tropical Agriculture and Human Resources
University of Hawaii

A SIMPLE TRANSFER CHAMBER FOR ASEPTIC WORK WITH MICROORGANISMS

Rhizobia are beneficial root-nodule bacteria which fix nitrogen in symbiosis with legumes. Isolation of rhizobia from root nodules, subculturing, plating, and other routine aseptic manipulations of these bacteria require a sterile environment. Commercially built laminar flow transfer hoods achieve this through air filtration and positive pressure within the chamber which prevents contaminants from entering. Unfortunately, these hoods are unaffordable for many researchers and teachers of *Rhizobium* microbiology in developing countries.

A simple chamber for aseptic work, as shown in Figure 1, can be constructed from readily available materials and according to the drawing in Figure 3 (detailed blueprints are available from the NifTAL Project). When using correct procedures and precautions, this chamber can produce good results, thus elimiating the need for expensively manufactured transfer hoods for routine work with *Rhizobium*.

In this design, the placement and position of the bunsen burner is critical for producing a sterile environment (Figure 2). The bunsen burner is admitted into the chamber through a hole made in the base in such a way that approximately one inch of the top of the burner protrudes into the chamber. Thus, the gas supply line and the air intake ports of the burner remain positioned outside the chamber. When the burner is lit, the flame eventually warms up the air inside the chamber and an unidirectional air current results. This warm air current exits through the open front thus preventing entry of contaminants.



R. Gabrielson

Figure 1. Transfer Chamber in Use

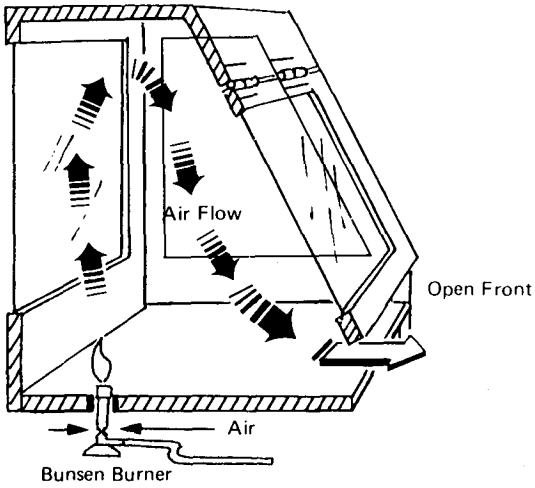


Figure 2. Cross Section of Chamber Illustrating Working Principle

Construction:

The dimensions are given in Figure 3 and materials used are readily available in most hardware stores. Back, top, bottom, front door frame, and sides are made of plywood (1.5 cm thick). Window openings are cut out and plexiglass or glass is cut to size to fit the openings. Hardwood moldings are used to hold the glass in place. A wooden reinforcement strip across the top of the chamber serves as an anchor for the door which is attached to it by three cabinet door hinges. Plastic laminate is glued over the working surface with contact cement. A 1.5 cm hole is drilled at 14 cm distance from the back of the chamber and at an equal distance from each side. The chamber is elevated by four wooden blocks which are glued into place. All uncovered wooden surfaces are painted with an oil-based or epoxy-type paint.

Operating Instructions:

1 Open the hinged door and wipe the interior thoroughly with an antiseptic such as 70% ethanol. Allow the ethanol to dry.
2 Turn on the gas and light the burner. The flame should be blue and adjusted to no more than 6 cm high.
3 Close the hinged door and wait 10 minutes before using the chamber.
4 When work is completed, turn off the flame and disconnect the burner gas line. This is important because a leaky gas valve may cause the chamber to fill with gas possibly causing an explosion when the burner is lit again for operation.

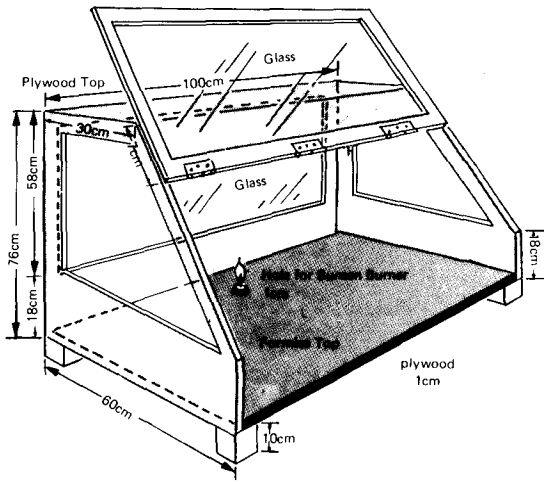


Figure 3. Construction Drawing of Chamber

by Heinz J. Hoben and Padma Somasegaran

*The NifTAL Project (P. O. Box O, Paia, Maui, HI 96779) is an agricultural research project funded by USAID.