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Practical methods for the quality control of inoculant biofertilisers

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ACIAR

2011

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Deaker R., Kecskés M.L., Rose M.T., Khanok-on Amprayn, Ganisan Krishnen, Tran Thi Kim Cuc, Vu Thuy Nga, Phan Thi Cong, Nguyen Thanh Hien and Kennedy I.R. 2011. Practical methods for the quality control of inoculant biofertilisers. ACIAR Monograph No.147. Australian Centre for International Agricultural Research: Canberra. 101 pp.

ACIAR Monograph No. 147
ISBN 978 1 921738 83 8 (print)
ISBN 978 1 921738 84 5(online)

Technical editing by Biotext, Canberra
Design by Zoo Advertising
Printing by BlueStar Print

Foreword

Research supported by the Australian Centre for International Agricultural Research (ACIAR), the Australian Agency for International Development (AusAID) and the World Bank has resulted in this published set of protocols and guidelines for the quality control of inoculant biofertilisers that are applied to rice by Vietnamese farmers. The choice of methods selected for the routine testing of biofertiliser quality seeks a balance between the use of modern technologies and the needs of developing countries with limited resources other than labour.

Inoculant biofertilisers are preparations that contain beneficial micro-organisms and are applied to crops with the aim of reducing or replacing more costly and environmentally damaging chemical fertilisers. In previous work supported by ACIAR and AusAID, rice yields in Vietnam could be maintained and in some cases increased with reduced application rates of chemical fertiliser, particularly nitrogen, when treated with the biofertiliser, BioGro. With reduced input costs and the potential for increased yields, farmers could gain substantial benefits from biofertiliser application to cereals.

However, these benefits can only be achieved if adequate quality control of biofertiliser production and application is available. Quality control should verify that there are sufficiently high viable numbers of the correct strains of micro-organisms present in the biofertiliser over the shelf life of the product.

Farm trials should also be part of the quality control, proving that the biofertiliser actually promotes crop yield with reduced chemical fertiliser inputs. Biofertiliser technology should help to generate wealth cooperatively in local communities, reducing the need for more expensive manufactured products, such as nitrogenous fertiliser. Similar developments may occur in farming communities elsewhere in the next decade and this work in Vietnam has the potential to provide valuable insight.

Similar positive results on crop yields have been obtained following inoculation of rice with other biofertiliser strains in Pakistan and with inoculation of maize in the United States, Mexico and Argentina. Other crops such as sugarcane have also benefited from associative micro-organisms.

Inoculant micro-organisms suitable for inclusion in biofertilisers for cereal crops, such as those described here, are predicted to have an important role to play in sustainable agriculture in the 21st century. However, this will only come about if the highest standards of quality control are maintained in their production.

Nick Austin
Chief Executive Officer
ACIAR

Acknowledgments

We wish to acknowledge the financial support of the Australian Centre for International Agricultural Research, the Australian Agency for International Development (AusAID) and the World Bank's Development Marketplace.

Throughout the period of research, Dr Rodney Roughley, Director of the Australian Inoculant Research and Control Service (AIRCS) from 1970–76 and again from 1980–1996, has provided valuable guidance, criticism and advice, drawing on his extensive experience with the quality control of legume inoculants in Australia.

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Abbreviations

Acronyms

abs	absorbance
ACC	1-aminocyclopropane-1-carboxylate
ACIAR	Australian Centre for International Agricultural Research
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FYM	farmyard manure
HRP	horseradish peroxidase
IgG	immunoglobulin G
MNA	modified nutrient agar
MNB	modified nutrient broth
MPN	most probable number
NA	nutrient agar
P	phosphorus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	real-time PCR
rDNA	ribosonal DNA
RNA	ribonucleic acid
TAE (buffer)	tris-acetate-EDTA
TMB	3,3',5,5' tetramethylbenzidine
TMPD	tetramethyl-r-phenylenediamine
TSA	trypticase soy agar

Chemical names

$(NH_4)_2SO_4$	ammonium sulfate
$Ca_3(PO_4)_2$	tricalcium phosphate
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
CO_2	carbon dioxide
$FeCl_3$	iron chloride
$FeSO_4$	iron sulfate
$FeSO_4.7H_2O$	iron sulfate heptahydrate
H_2O_2	hydrogen peroxide
H_2SO_4	sulfuric acid
HCl	hydrochloric acid
I_2	iodine
K_2HPO_4	dipotassium hydrogen phosphate
KCl	potassium chloride
KH_2PO_4	monobasic potassium phosphate
KI	potassium iodide
KOH	potassium hydroxide
$MgSO_4.7H_2O$	magnesium sulfate heptahydrate
$MnSO_4$	manganese sulfate
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium hydrogen phosphate
Na_2MoO_4	sodium molybdate
NaCl	sodium chloride
$NaH_2PO_4.H_2O$	sodium dihydrogen phosphate
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NaOH	sodium hydroxide
NO_3	nitrate

Units

µg	microgram
µL	microlitre
µm	micrometre (or micron)
atm	atmosphere
cfu	colony forming units
cm	centimetre
d	day
g	gram
h	hour
K	kelvin
L	litre
m^2	square metre
M	molar
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mmol	millimol
mol	mole
N	normal
ng	nanogram
nL	nanolitre
nm	nanometre
nmol	nanomole
pmol	picamole
ppm	parts per million
rpm	rotations per minute
s	second
STP	standard condition for temperature and pressure
T_0	time zero (start of time course experiment)
V	volt

Introduction

BioGro is a multistrain plant growth-promoting biofertiliser that has been the subject of several collaborative research projects in Australia and Vietnam since the late 1990s. There is now clear evidence that BioGro is beneficial to rice farmers in Vietnam. Reports of the effects of BioGro on rice growth and yield, with decreased chemical inputs, and economic and commercial assessments of application have already been published (Nguyen et al. 2003; Kennedy et al. 2008; Phan et al. 2008). One of the major features of this research was the development and implementation of quality control protocols to ensure consistency in the production and application of BioGro throughout the research and for ongoing production in Vietnam.

This book is intended to be used as a guide to setting up a system for quality control of biofertilisers, and as a manual of laboratory and field-based quality control protocols. It summarises our laboratory and field experiences with biofertilisers, research supported by the Australian Agency for International Development and ACIAR, since the Sydney University Centre for Nitrogen Fixation (SUNFix) began collaborating with Vietnamese colleagues, in the late 1990s.

The book describes methods for the isolation, selection, identification and enumeration of biofertiliser strains, and quality control of inoculant biofertiliser products in the laboratory and field. It includes methods for demonstrating plant growth promotion, molecular techniques such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and immunoblotting, and cultivation of starter cultures for the production of high-quality commercial products.

Not all the methods described in the book need to be applied in any particular laboratory, or all the time. On the contrary, each laboratory should develop its own most convenient subset of tests, designed to suit local needs and conditions.

Sections 1–3 bring together a range of methods for the functional analysis of biofertiliser strains. Some of these will need to be applied for a particular organism or strain only once, such as the molecular methods related to ribosomal deoxyribonucleic acid (DNA) used in the specific identification of strains. As such, the more advanced molecular techniques are not needed on a routine basis and could be applied in collaborating laboratories or even in other countries, as was the case with the linkage to SUNFix. This strategy has the advantage of expanding the range of expertise available and allows a greater number of laboratory or field workers to become involved.

Sections 4 and 5 deal with methods required on a day-to-day basis either in preparing high-quality biofertiliser inoculants or in showing their effectiveness in the field. The appendixes include some real examples of data records and reports used in the quality control of biofertilisers in Vietnam.

Users of this book can learn from our experiences and adapt the most appropriate set of methods for their own needs, or develop their own set of improved tests. Feedback to the authors is welcome.

01

Selection and functional characterisation of plant growth-promoting inoculant strains

The first step in developing an inoculant biofertiliser product is the selection and characterisation of microbial strains that promote plant growth. Growth promotion can be defined as the overall increase in biomass of inoculated plants compared with a population of uninoculated plants. The exact cause of plant growth promotion is often difficult to define as it may arise via a number or combination of direct or indirect mechanisms.

One direct mechanism that micro-organisms use is increasing plant nutrient uptake by affecting the production or regulation of plant hormones (e.g. auxin), or affecting ACC (1-aminocyclopropane-1-carboxylate) deaminase activity and ethylene production—which in turn increase root branching and length, effectively increasing surface area. They also affect cell elongation, cell membrane permeability and the plant's responses to stress.

Another direct mechanism is to increase nutrient availability through the fixation of atmospheric nitrogen gas (N_2) and the mobilisation of inorganic and organic nutrients, including nitrogen, phosphorus (P), sulfur and micronutrient metals, or the production of siderophores that increase nutrient iron and zinc uptake.

Indirect mechanisms include antagonism towards phytopathogens and synergism or mutualism with beneficial co-inoculated or naturalised soil micro-organisms.

Certain bacteria may indirectly enhance these mechanisms by protecting or helping those bacteria or fungi that are directly involved in plant growth promotion. Mutualism with rhizobial or mycorrhizal strains can be especially important. A more in-depth discussion of all of these processes is given in a review by Vessey (2003).

Potentially useful strains can be isolated from plant rhizospheres exhibiting increased seedling growth. When purified they may be characterised according to their particular mechanism of action. Alternatively, pure cultures can be selected from rhizospheres by specific plant growth-promoting function based on functional selective media, and subsequently tested individually or in combinations for any plant growth-promoting activity. Similar approaches can also be taken to isolate endophytic strains, taking care to ensure adequate surface sterilisation to avoid contamination and/or misrepresentation (Hallman et al. 1997).

Following enrichment and isolation, functional characterisation of potential strains is necessary for a number of reasons. Firstly, functional characteristics can be used as a non-specific quality-control factor for the biofertiliser inoculant, as they are often quicker and cheaper than strain-specific tests. Secondly, knowing the pathway by which biofertiliser strains promote plant growth allows the potential of the inoculant to be optimised under different agronomic conditions. For example, a biofertiliser containing a calcium phosphate solubilising bacteria could theoretically be recommended with an appropriate P fertiliser; N_2-fixing biofertilisers may be supplemented with the molybdenum required by nitrogenase in molybdenum-deficient soils; or a pathogen-inhibiting

biofertiliser may be used in rice fields suffering growth decline caused by that pathogen. Thirdly, strains may be mixed in suitable combinations that target multiple plant growth-promoting pathways, or assist in each other's survival and action.

Apart from their specific plant growth-promoting function, a number of additional selection criteria for biofertiliser micro-organisms have been recommended (Nguyen et al. 2003). These included that the strain:

- should be generally abundant in the rhizosphere from which it was isolated
- should be easily cultured and fast growing, to improve the success rate if a non-sterile carrier is to be used
- must be shown not to cause root disease or inhibit other beneficial micro-organisms
- must not be a risk to human health.

Although it is difficult to claim that the isolated strain is dominant in the rhizosphere, this criterion applies with reference to the relative abundance after enrichment and isolation on microbiological media. All potentially successful inoculant micro-organisms must initially be isolated and grown on culture media; however, metagenomic approaches may be useful to determine abundant, non-culturable populations. Specific media may then be better designed to isolate and culture identified species.

1.1 Increased seedling growth

Testing for increased seedling growth is very important because the early response of plants to biofertilisers appears to be the primary indicator of biofertiliser effect. Genetic stability of the plant growth-promoting effect is critical to the success of biofertilisers and may be affected by the way in which strains are preserved.

Strain stability should be tested regularly—at least yearly or before their release for manufacture—by monitoring the effect of the biofertiliser on the growth of seedlings of the target crop. Records should be kept so that any changes from year to year can be observed. If plant growth promotion is not observed, strain identity should be confirmed followed by tests for maintenance of plant growth-promoting mechanisms, such as nitrogen fixation, phosphate mobilisation and plant hormone production. However, the effect of variable plant growth conditions (see box on following page) must always be considered during testing, and the growth of inoculated plants should always be compared with uninoculated control plants.

1.1.1 Reagents and equipment

- Sterile deionised water
- 10% H_2O_2
- Watanabe (1959) medium—3 g agar; 5 g glucose; 0.1 g yeast extract; 0.15 g $CaCl_2$; 0.005 g Na_2MoO_4; 0.2 g $MgSO_4.7H_20$; 0.04 g $FeSO_4.7H_20$; 0.5 g K_2HPO_4; 5 mL bromothymol blue (0.5% alcohol solution); 1.0 g $CaCO_3$; 1,000 mL deionised water; pH 7.0
- Sacra agar—1.0 g KNO_3; 0.5 g $CaSO_4.2H_20$; 0.5 g $MgSO_4.7H_20$; 0.5 g NaCl; 0.5 g $Ca_3(PO_4)_2$; 5 g glucose; 1,000 mL deionised water; 1 drop $FeCl_3$ (5% solution); 5 g agar
- Washed sand and Ca_3PO_4

1.1.2 Sample preparation

1. Shake rice seeds in sterile deionised water for 15 min.
2. Filter, then soak seeds in 10% H_2O_2 for 15 min.
3. Wash seeds again, with shaking, in three changes of sterile deionised water.

4. Transfer seeds to Petri dishes containing Watanabe medium prepared with 0.3% agar for germination.
5. After 2 days, transfer the germinated seeds to Sacra agar in large test tubes or in 500 mL Erlenmeyer flasks; inoculate the seedlings with the test strain of biofertiliser and grow them for 10 days at 25 °C. Germinated seedlings may also be transferred after 2–3 days to pots (350–450 mL capacity) containing washed sand and 0.5 g Ca_3PO_4 and inoculated with the test strain. Ten seedlings may be planted per pot, thinned to five after 5 days and grown for 4 weeks. For each strain or combination of strains, inoculate 18 pots arranged in three replicates of 6 pots.
   Visually assess the shoots and roots of the seedlings and then dry and weigh them. Observations from inoculated plants should be compared with uninoculated controls.

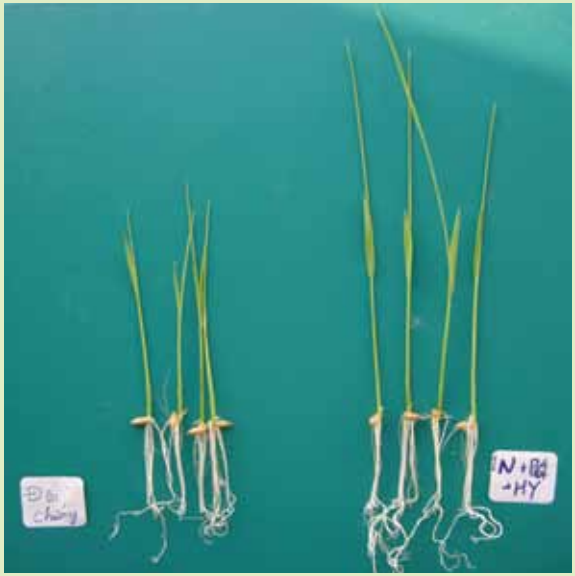
1.2 Nitrogen fixation (acetylene reduction assay)

Nitrogen fixation by free-living or associative micro-organisms can have a significant influence on plant nutrition. The nitrogen-fixing capabilities of biofertiliser strains should be quantified when they are isolated and periodically after that to ensure they maintain this capability.

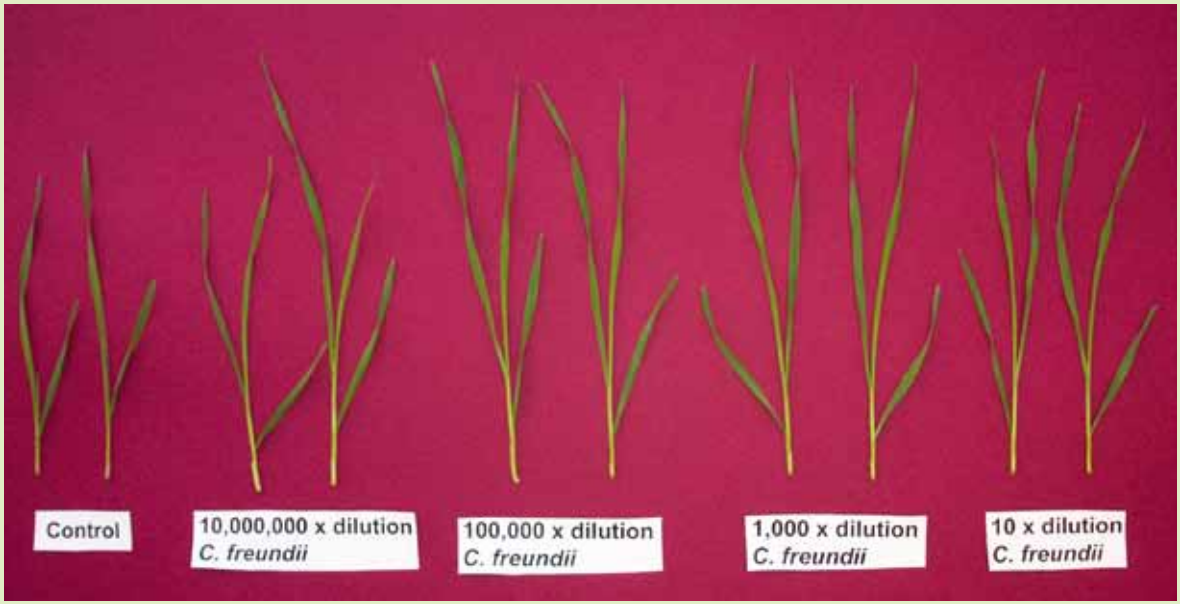
This method works on the basis that the nitrogenase enzyme, which reduces N_2 to ammonium (NH_4^+), also reduces acetylene to ethylene (Dilworth 1966). Cultures of the test strain are grown in enclosed vessels and the production of ethylene from acetylene is measured using gas chromatography (Stewart et al. 1967; but see Staal et al. 2001 for recent methodology).

Testing biofertiliser strains

It is important to test strains for their ability to improve plant growth and compare them with uninoculated controls. Using a system of growing rice seedlings in sterile sand, the positive effects of BioGro (a biofertiliser developed in Vietnam) could be observed. However, testing for seedling-growth promotion by micro-organisms may have variable effects under different environmental conditions. For example, micro-organisms that are plant growth-promoting under some conditions may suppress growth or act as pseudo-pathogens under different conditions.



Effect of BioGro on the growth of rice seedlings (right); in comparison, control plants (left) had smaller roots and shoots.



Effect of increasing numbers of Citrobacter freundii *(3C) on wheat shoot growth 10 days after planting.*

Such an effect was observed by Rodrigues et al. (2008) when they inoculated rice seedlings with a number of different *Azospirillum* strains under gnotobiotic conditions in growth cabinets. The authors observed reduced growth of both shoots and roots, suggesting that it was due to excess phytohormone accumulation in the medium. When the same strains were inoculated onto rice seedlings grown in the glasshouse under non-sterile conditions, increased growth, nitrogen accumulation and grain yield were all observed.

Ideally, plant-growth assays should occur in conditions that do not limit growth. Transient effects of *Citrobacter freundii* (3C) on wheat growth were observed at 10 days after planting in a study at the University of Sydney. Control plants had only two leaves and all inoculated plants had three leaves (see above photo). Differences between treatments had disappeared at 15 days. In this case, it is likely that root growth was limited by pot size. Inhibitory effects on growth may also be amplified by restricted growing environments. Although not statistically significant, plants that had been inoculated with 2.6×10^8 colony forming units had slightly reduced growth compared with plants inoculated with smaller inoculum sizes.

Protocols for measuring the effect of ACC deaminase activity on plants are outlined in Penrose and Glick (2003).

1.2.1 Reagents and equipment

- Microbial culture in semi-solid agar or other broth
- Acetylene and ethylene gases with gas bladders
- Glass serum or McCartney bottles (30 mL) and rubber serum stoppers (Thomas, Chicago)
- 1 mL and 5 mL plastic syringes and needles
- Flame ionisation detector (FID) gas chromatograph (GC)

1.2.2 Reaction preparation

1. Set up the GC (e.g. Shimadzu GC8A) with gases flowing at the correct rate to ensure the detector flame remains alight (check with shiny surface for condensation from the burner outlet); set correct pressure points on the GC dials for future reference.
2. Optimise the GC oven and detector conditions: Waters 1 m glass Poropak T column, temperature 100 °C and FID temperature 150 °C.
3. Adjust gas flow parameters so the ethylene peak (100 ppm, dilute 1:10,000 by volume) registers between 1 min and 2 min, and the acetylene peak registers between 2 min and 5 min, injecting authentic standards. Inject standards (0.5 mL or 1.0 mL, smoothly and rapidly, avoiding back flushing through the syringe, until reproducible peaks are obtained. A 1:10,000 dilution contains about 2 nmol ethylene per mL, given 24.45 nL = 1 nmole at standard condition for temperature and pressure (STP; 298 K and 1 atm).
4. The amount (volume) of biomass required depends on the thickness of cultures. Volume can be increased and larger volume serum flasks can be used depending on resulting peaks.
5. For liquid cultures, add 10 mL of culture to the serum bottle and seal with good-quality rubber serum stoppers; inject 2–3 mL as 10% of flask volume (acetylene is highly soluble in water, and equal concentrations will occur in the aqueous fraction and the head space). Shake well for 30 s to initiate reaction and inject a zero time control. Acetylene usually contains a small methane peak eluted first and a trace of ethylene.
6. Incubate under ambient conditions for 1–2 h, taking 0.5 mL samples at 15 or 30 min intervals.
7. Read peak area or height and retention times and calculate acetylene reduction over different times from T_0.

1.3 Phosphorus mobilisation

Phosphorus mobilisation by biofertiliser micro-organisms may increase P availability in the rhizosphere, leading to increased plant uptake or indirect stimulation of beneficial rhizo-micro-organisms. Phosphorus mobilisation may result from the excretion of H cations, organic anions or phosphatase/phytase enzymes (Illmer and Schinner 1995; Vassilev et al. 2006). The potential for P-mobilising organisms to promote plant growth can be estimated by laboratory testing (Harris et al. 2006). The following protocol provides a framework for quantifying these aspects of insoluble P mobilisation.

1.3.1 Reagents

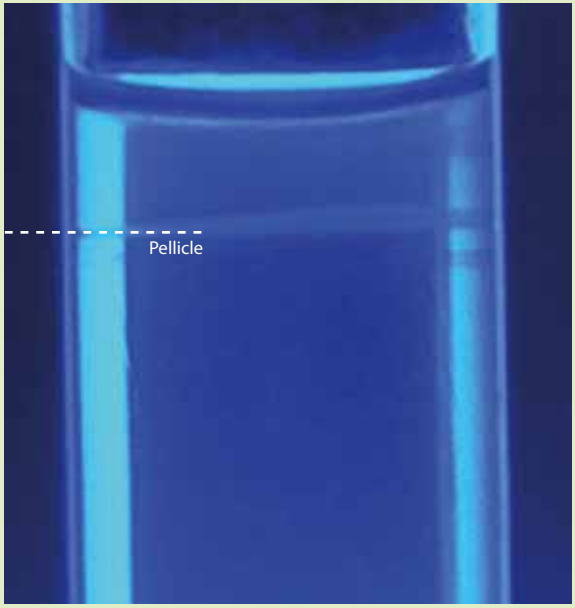
The medium used for growing P-solubilising micro-organisms will vary according to specific requirements. Generally, media containing very low or no added soluble P is supplemented with insoluble or sparingly soluble sources of P. Zones of clearing may be observed

Using the rising pellicle assay for nitrogen fixation

The environmental conditions inhabited by the microbial strain of interest will strongly affect potential rates of nitrogen fixation. Because the nitrogenase enzyme is oxygen sensitive, yet requires high amounts of energy, the level of oxygen is of critical importance (see Gallon 1992 for a review). A preliminary test for nitrogen fixation by some aerotaxic motile bacteria is the presence of a 'rising pellicle' in semi-solid, nitrogen-free agar tubes. The bacteria will move to a depth in the vertical tube that corresponds to a level of dissolved oxygen that is optimum for both aerobic respiration (energy production) and nitrogen fixation. As the oxygen is consumed at depth, the pellicle will rise toward the higher oxygen concentration at the surface as the bacteria show aerotaxis.

The addition of an acid–base indicator to nitrogen-free culture media can also assist in the presumptive identification of nitrogen fixation. Nitrogen fixation is a reductive process, requiring hydrogenation of dinitrogen. It results in a net uptake of hydrogen ions and the subsequent alkalinisation of the cell can be detected by an indicator such as bromothymol blue, which changes from yellow in acidic media to blue in alkaline media with an endpoint pH of 7.1.

If molecular biology facilities are available, it is also possible to directly determine if certain genes coding for nitrogenase activity are present in plant growth-promoting isolates by polymerase chain reaction (PCR). There are a number of specific primers available, but the nitrogenase reductase gene *nifH* is the most commonly targeted (Poly et al. 2001; Diallo et al. 2008). The protocol given in Section 2.3 can be adapted for this purpose. It is important to note that presence of the gene does not indicate nitrogen fixation; gene presence is only an indicator of the potential for nitrogen fixation. Transcription of the gene to mRNA is a better indicator of nitrogen-fixing potential.



Rising pellicle of Azospirillum brasilense *in a nitrogen-free, semi-solid agar medium with malate (Baldani and Döbereiner 1980). The pellicle rises as oxygen is consumed and the level corresponds to the optimum dissolved oxygen for nitrogen fixation.*

as evidence of P solubilisation on solid media or soluble P may be measured in liquid media over time using methods outlined below (see Figure 1.1). It is important to note that timing of the measurement of soluble P in media is important as it will vary with the growth stage of the micro-organisms. Cells use P for their own growth and P is released after cell lysis.

Media used for growth may contain crude extracts such as soil (Illmer and Schinner 1995) or yeast (Pikovskaya 1948), or be fully defined (Beck and Munns 1984). Crude extracts may contain unknown amounts of soluble P.

- Liquid or solid P-free growth media (which ideally should be buffered)
- Insoluble or sparingly soluble phosphorus compounds
- 1 M NaOH and 1 M HCl (for adjusting pH)
- Specific reagents as indicated in each method

1.3.2 Microbial culture

1. Prepare reagents and media.
2. Prepare fresh cultures on agar to be used for inoculation.
3. Prepare treatment flasks containing liquid media for inoculation:
   – Mix media and add P source. After mixing and autoclaving media and P source, check the pH. P source and media may be autoclaved separately before mixing (as in Illmer and Schinner 1995). Media can be buffered using zwitterionic buffers (Good et al. 1966; Good and Izawa 1972) and the pH adjusted using HCl or NaOH.

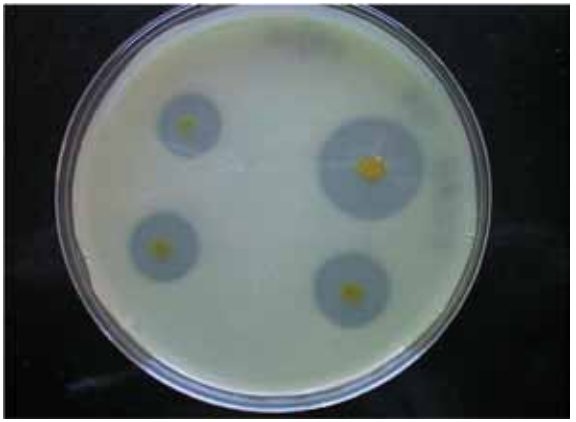


Figure 1.1 *Phosphate-solubilising* Klebsiella pneumoniae *4P. Zones of clearing around bacterial colonies indicate solubilisation of insoluble phosphorus in the medium.*

4. Take samples and determine the initial soluble P concentration (T_0).
5. Inoculate flasks with a known number of colony forming units (cfu) of each strain. (It is desirable to inoculate with a low number of cells to reduce the introduction of soluble P contained within cells.)
6. Incubate flasks at 30 °C with shaking.
7. Sample from each flask at various times and measure the viable cell number, soluble P, organic acid and phosphatase activity (see protocols below).

1.3.3 Soluble phosphate assay

(molybdenum blue method, modified from Murphy and Riley 1962)

Reagents

- Molybdate reagent—prepared by mixing two solutions. For the first, dissolve 8.5 g of ammonium molybdate in 600 mL of deionised water and slowly add 99.2 mL of concentrated H_2SO_4. For the second, dissolve 0.196 g of potassium antimony tartrate in 100 mL of deionised water. When both reagents are completely dissolved, mix the two solutions together in an amber glass bottle and store at 4 °C.
- Boric-tartaric acid diluent—dissolve 36 g of boric acid and 2.4 g of tartaric acid in deionised water and make up to 1 L.
- Ascorbic acid reagent—dissolve 0.875 g of ascorbic acid in 100 mL of molybdate reagent. This solution should be made fresh for each day of analysis.
- Stock phosphate standard solution (1 mM)—dissolve 0.1361 g of dry (65 °C for 72 h) KH_2PO_4 in 800 mL of deionised water then make up to 1 L in a volumetric flask. Store in a dark bottle with 1 mL of chloroform.

Procedure

1. To determine soluble P, centrifuge cultures and solutions containing insoluble P at 20,000 × g for 10 min and analyse the P content of the supernatant. The assay can be carried out in 1.5 mL microfuge tubes using 0.1 mL supernatant and standard solution.
2. Transfer 0.1 mL supernatant to microfuge tubes.
3. Prepare a set of standards from 0 ppm to 30 ppm using the standard stock solution and transfer 0.1 mL to microfuge tubes.
4. Add the following reagents to all tubes in order: 0.8 mL boric-tartaric acid diluents, 0.1 mL molybdate reagent and 0.1 mL ascorbic acid reagent. Mix thoroughly with a vortex mixer.
5. Place tubes in a water bath at 80 °C for 10 min so that the water is at the same level or above the contents of the tube.
6. After removing tubes from the water bath, allow to cool for 10 min and read the absorbance using a spectrophotometer at a wavelength of 660 nm.
7. Draw a calibration curve from the standard readings and use it to calculate the amount of P in the samples. If the readings do not fall within the standard curve, dilute samples and run the reaction (including standards) again.
   Note: Absorbance readings should not be higher than the limit of the spectrophotometer (i.e. ~1.2). If absorbances of standard solutions are above this, dilute and repeat reaction.

1.3.4 Phosphatase assay

Reagents

- Modified universal buffer (MUB) stock solution—dissolve 12.1 g of tris(hydroxymethyl)-aminomethane (THAM), 11.6 g of maleic acid, 14 g of citric acid and 6.3 g of boric acid in 488 mL of 1 M NaOH and dilute the solution to 1 L with deionised water. Store at 4 °C.
- MUB working solutions (pH 6.5 and pH 11)—place 200 mL of MUB stock solution in a 400 mL beaker containing a magnetic stirring bar and place the beaker on a magnetic stirrer. Titrate the solution to pH 6.5 with 0.1 M HCl and adjust the volume to 1 L with deionised water. Titrate another 200 mL of the MUB stock solution to pH 11 by using 0.1 M NaOH and adjust the volume to 1 L with deionised water.
- p-nitrophenyl phosphate (PNPP) solution (0.05 M)—dissolve 0.840 g of disodium PNPP tetrahydrate in about 40 mL of MUB pH 6.5 or pH 11, and dilute the solution to 50 mL with MUB of the same pH. Store at 4 °C.
- $CaCl_2$ (0.5 M)—dissolve 73.5 g of $CaCl_2.2H_2O$ in 1 L of deionised water
- NaOH (0.5 M)—dissolve 20 g of NaOH in 1 L of deionised water
- p-nitrophenol (PNP) standard solution (1 g/L)—dissolve 1 g of PNP in 1 L of deionised water. Store at 4 °C.

Procedure

1. Into each test tube, pipette 4 mL of MUB, 1 mL of PNPP solution and 0.5 mL of culture supernatant. Vortex to mix.
2. Stopper and incubate at 37 °C for 1 h. Monitor the tubes during incubation and stop reaction by adding 1 mL of 0.05 M $CaCl_2$ and 4 mL of 0.5 M NaOH if yellow colour develops, making a note of the time.
3. After 1 h, remove all tubes and stop the reaction by adding 1 mL of 0.05 M $CaCl_2$ and 4 mL of 0.5 M NaOH. Vortex to mix.
4. Include a control containing culture supernatant with each run, but add PNPP to the tubes after adding $CaCl_2$ and NaOH before incubation.
5. Measure the intensity of the yellow colour at 410 nm in a spectrophotometer.
6. Calculate the PNP content of the solution by reference to a calibration curve plotted from results obtained from standards of 0, 10, 25, 50, 75 and 100 µg. Express results as nmoles PNP/cfu/min.

Use this procedure for both acid and alkaline phosphatases.

1.3.5 Organic acid assay

This assay depends on the set-up of high-performance liquid chromatography (HPLC) being used, in particular the type of column (e.g. an anion exchange column or a C18 reversed-phase column). A UV detector is necessary.

Reagents

- Dowex 50W–X8 exchange resin (BDH; 20–50 US mesh)
- Concentrated phosphoric acid and HCl
- Ethyl acetate
- Mobile phase: 10 mM KH_2PO_4 – CH_3OH (95:5), pH 2.7–3.0
- 10 mM organic acid standards

Procedure

1. Centrifuge 25 mL of bacterial culture at 3,600 × g for 5 min and shake 5 mL of the supernatant with Dowex 50W–X8 exchange resin to remove salts.
2. Filter 3 mL of treated supernatant through a 0.45 µm nitrocellulose membrane into a 4 mL HPLC vial.
3. If chromatograms are not clear (too many interfering peaks) or if organic acid concentrations are below the limit of detection, the sample may need extraction/concentration/clean-up, using steps 4–6.
4. Acidify 20 mL of supernatant to pH 2.5 with 1 M HCl.
5. Extract the acidified supernatant by using 5 mL of ethyl acetate three times, drawing off the ethyl acetate from the top each time. Combine the extracts.
6. Evaporate the solvent to dryness in a rotary evaporator at 40 °C. Redissolve the residue in 2 mL of deionised water.

HPLC analysis

1. Set the detector wavelength to 215 nm and the mobile phase (I) pumping at 0.5 mL/min for a Polypore H column, OR mobile phase (II) pumping at 0.8 mL/min for a C18 column (4.6 mm × 250 mm × 5 µm).
2. Inject mixed organic acid standard (50 µL) and individual organic acid standards, and determine residence times for each acid.
3. Inject different masses of standard mixture to obtain a standard curve.
4. Inject treatment samples and quantify the type and amount of acid produced, using internal standards if necessary.

The Pho regulon in bacteria

A key genetic factor in uptake and homeostasis of phosphate in bacteria is the Pho regulon. It is controlled by a two-component regulatory system: the first component senses environmental inorganic phosphate levels and, under conditions of inorganic phosphate limitation, activates a partner response regulator that up-regulates or down-regulates numerous target genes (Lamarche et al. 2008). A global analysis of proteins synthesised in *Escherichia coli* under inorganic phosphate restriction showed that the Pho system could regulate more than 400 genes, comprising up to 10% of all genes present (Van Bogelen et al. 1996).

Potential phenotypic responses to variations in environmental inorganic phosphate concentration include changes in cell morphology, cell surface components, exopolysaccharide production for root invasion, endotoxin production, sporulation and acid-stress response mechanisms (Lamarche et al. 2008). One of the main opportunities for exploiting this reaction to promote plant growth is through increased pathogen inhibition. A good review on the potential and future trends for this area of research is given by Vassilev et al. (2006).

1.4 Indole acetic acid production

Indole-3-acetic acid is an auxin that has been implicated in plant growth promotion by stimulating root growth and increasing root branching. The following simple photometric protocol has been adapted from Gordon and Weber (1951).

1.4.1 Reagents

- Acidic iron reagent—add 25 mL of 12 M H_2SO_4 to 50 mL of deionised water. Add the acid slowly and **very carefully** as this dilution produces a large amount of heat. Following this, add 5 mL of 0.5 M $FeCl_3$ and mix well.

1.4.2 Indole acetic acid assay

1. Dissolve 0.05 g of analytical-grade indole-3-acetic acid in 100 mL of 50% ethanol:deionised water to make a 500 µg/mL stock solution.
2. Dilute 1 mL of the stock solution to 50 mL to make a 10 µg/mL solution. This is the intermediate stock solution.
3. Make a concentration series of indole acetic acid as outlined in Table 1.1, vortexing after addition of the reagent. From the results, prepare a standard curve.
4. Centrifuge 10 mL of broth culture (and sterile broth controls) at 3,600 × g for 5 min and pipette 2 mL aliquots of supernatant into sample tubes.
5. To each sample add 4 mL of acidic iron reagent and vortex.
6. Incubate in the dark for 45 min and then read at 525 nm.

Table 1.1 *Concentration series for indole acetic acid*

	Indole acetic acid standard (µg/mL)						
	0	**0.5**	**1.0**	**2.5**	**5.0**	**7.5**	**10.0**
Volume of intermediate stock (mL)	0	0.1	0.2	0.5	1	1.5	2
Volume of deionised water (mL)	2	1.9	1.8	1.5	1	0.5	0
Volume of Fe-H_2SO_4 reagent	4	4	4	4	4	4	4
Absorbance[a]	0	0.005	0.013	0.046	0.096	0.150	0.204

a Approximate absorbance values

02

Identification of plant growth-promoting inoculant strains

Identification of microbial plant growth-promoting isolates is extremely important for several reasons. Placing an isolate within an existing taxonomic group can provide a lot of information about conditions for growth and survival based on published information about other similar micro-organisms. This may also provide an insight into possible functional biochemical pathways and a means to re-isolate and confirm identity with more confidence.

There are several approaches to identifying micro-organisms and it is recommended that more than one approach be adopted. Where whole-cell techniques may indicate that cells have changed physiologically in response to environmental conditions, DNA sequencing can be used to confirm identity. Physiological or biochemical changes in cells are important to understand and may allow optimisation of conditions for their beneficial plant-growth functions.

2.1 Biochemical, morphological and physiological identification techniques

2.1.1 Reagents

- 2% crystal violet—solution A: 20 g of crystal violet in 200 mL of 95% ethanol; solution B: 8 g of ammonium oxalate in 800 mL of deionised water. Mix solutions A and B together and let stand overnight.
- Gram's iodine—2% I_2 and 3% KI in 70% ethanol; grind I_2 and KI crystals together in a mortar and pestle, slowly add deionised water while grinding. Solution should be a deep golden colour and will lighten with age. Aged, light-yellow solutions should be discarded.
- 95% ethanol or 50:50 ethanol:acetone solution
- Safranin—25 g safranin in 100 mL of 95% ethanol and 900 mL of deionised water
- 3% H_2O_2
- Tetramethyl-r-phenylenediamine (TMPD) or commercially available oxidase paper
- Microbiology media

2.1.2 Gram staining

Gram staining differentiates bacteria on the basis of their cell wall constituents. Gram-positive cells retain a purple crystal violet–iodine complex because of the high percentage of peptidoglycan in their cell walls, whereas gram-negative bacteria lose this purple coloured complex when washed with ethanol. Gram-negative cells are usually then counterstained with safranin to give a pink–red colour.

Gram stains can yield false results, particularly for bacteria recovered from environmental samples. Gram staining may be confirmed using KOH and vancomycin tests; however, reactions of 488 different strains indicated that false results were also possible with these tests (Von Graevenitz and Bucher 1983). As each test may yield false results, it is recommended that more than one test be used to confirm Gram reaction.

The formation of a zone of clearing around discs containing 5 µg vancomycin can be used to indicate gram-positive organisms. However, vancomycin susceptibility or resistance in individual strains may confound results. Gram-negative bacteria may be indicated by the production of 'string' after emulsifying a colony in a drop of 3% KOH on a glass slide for 60 s. The gram-negative wall is broken down by the KOH and the resulting suspension should be thick and stringy when the loop is pulled away from the slide.

Gram staining protocol

1. Clean a glass slide using an abrasive powder cleanser and prepare a smear of each strain by suspending a loopful of culture in a drop of deionised water on the slide and allowing it to dry.
2. Fix the cells on the slide by passing it several times through the flame from a Bunsen burner.
3. Stain the cells using the following staining and washing steps:
    a. Cover the heat-fixed smear with a 2% crystal violet solution for approximately 1 min then wash gently with deionised water.
    b. Apply Gram's iodine to the smear for approximately 1 min then wash gently with deionised water and drain excess water from the slide.
    c. Decolourise the stain with 95% ethanol—hold the slide at an oblique angle over a light background and pipette the alcohol so that it flows over the smear. Excess crystal violet will drain from the smear as a purple discharge. Continue until the purple discharge stops. A mixture of ethanol and acetone (50:50) can also be used to decolourise stained smears.
    d. Stop the decolourisation process by washing gently with deionised water.
    e. Cover the decolourised smear with dilute fuchsin for 1 min or safranin for 3–5 min.
    f. Wash gently with deionised water, blot dry and examine under the microscope.

2.1.3 Motility

Examine a young broth culture of the organism microscopically using a 'hanging drop' preparation. Cells can become attached to glass by flagellae and may have a circular motion, resulting in a false negative. A hanging drop minimises the area of contact with the glass cover slip. Motility should be distinguished from Brownian motion and streaming (usually observed in wet mount preparations) by observing the independent movement of cells around the field of view.

Hanging drop preparation

1. Place small but thick spots of petroleum jelly on each corner of a cover slip using a wooden tooth pick.
2. Thoroughly mix a log-phase broth culture and transfer a drop of culture onto the centre of the cover slip. Do not spread the drop around, leave it as a small drop.
3. Lower a slide gently to make contact with the petroleum jelly, but not the drop of culture.
4. Invert the slide and cover slip so that the drop hangs but does not come into contact with the slide and examine under the microscope.
5. To determine motility, ensure that cells observed are free from the glass cover slip. To do this, focus up and down to observe the full depth of the field of view.

Note: Motility may also be obtained from an oxidation-fermentation test (see Section 2.1.8).

2.1.4 Growth in air

Check for the ability of the organism to grow on culture media without reducing substances or anaerobic incubation. Grow cultures on agar in air —if colonies form, the culture is aerobic.

2.1.5 Growth in anaerobic conditions

The knowledge that an organism is unable to grow under anaerobic conditions may be diagnostically important. Some may be strict aerobes and others may need CO_2. Grow cultures on agar in an anaerobic jar (note that specialised anaerobic media may be required for growth). Oxygen in the jar should be removed by vacuum followed by flushing with CO_2. Alternatively, commercial powders containing iron, citric acid and sodium carbonate are available to remove oxygen and replace it with CO_2. The jar may then be incubated at the appropriate temperature.

2.1.6 Catalase test

Hydrogen peroxide is a harmful by-product of many metabolic reactions. The catalase enzyme is present in many organisms and catalyses the hydrolysis of hydrogen peroxide to oxygen and deionised water.

1. Place 1 drop of 3% H_2O_2 on a clean slide.
2. With a sterile loop, remove a large loopful of the organism to be tested from the plate or slope. Suspend the loopful on the drop of H_2O_2.
3. Examine the preparation for the presence of small bubbles of oxygen. A catalase-positive organism will have the enzyme capable of catalysing the hydrolysis of H_2O_2 to H_2O and O_2.

2.1.7 Oxidase test

Cytochrome oxidase catalyses the oxidation of cytochrome c and is widely distributed in nature. Oxidase activity can be detected through the use of redox reagents such as TMPD. Oxidation of TMPD results in a change from colourless to dark purple. The visible colour change is improved by impregnating white filter paper with TMPD. Oxidase reagent papers can be prepared in the lab by moistening sterile filter paper with sterile reagent. Alternatively, commercially prepared papers may be purchased from laboratory supply companies. Although there are advantages in using pre-prepared papers, the decision to purchase these will depend on the turnover of supplies, as shelf-life may be limited.

1. Place oxidase paper on a glass slide.
2. Smear the culture being tested across the impregnated paper using a sterile toothpick (do not use an inoculating loop for this test as it may produce a false positive result).
3. Discard the stick immediately into disinfectant.

Note: A positive reaction is indicated by a dark-purple colour developing on the paper within 10 s of inoculation.

2.1.8 Oxidation-fermentation (O-F) test

Micro-organisms can be classified by their ability to ferment (produce acid) from carbohydrates aerobically, anaerobically or under both conditions. This can be easily detected after observing growth and pH change in a semi-solid medium containing glucose, peptone and pH indicator. The culture is introduced to the medium in a test tube using a straight wire. Growth and pH change at different depths indicates oxidative or fermentative metabolism and relates to oxygen concentration.

O-F medium
(prepared in test tubes; Hugh and Leifson 1953)

- 2 g peptone (pancreatic digest of casein)
- 5 g NaCl
- 0.3 g K_2HPO_4
- 3 g agar
- 0.03 g bromothymol blue (1 g dissolved in 100 mL deionised water and 3 mL added to 1 L of medium)
- 10 g carbohydrate (glucose)
- pH 7.1

1. Inoculate a tube of O-F medium (Hugh-Leifson's medium) by stabbing with straight wire to within 1 cm of the bottom of the tube.
2. Incubate as necessary (2–14 d).

Note: This test determines whether an organism metabolises glucose oxidatively (i.e. in the presence of oxygen) or fermentatively (i.e. in the absence of oxygen).

Results are read as follows:

Results	Conclusion
Yellow at top of tube	Oxidation
Yellow throughout medium (sometimes with gas)	Fermentation
Blue or turning blue at top of tube	No action on carbohydrate

Notes:

1. A two-tube test may also be carried out. In this case, one tube is overlaid with mineral oil to produce anaerobic conditions.
2. Often additional information can be obtained from the O-F tube (e.g. motility and the production of gas from the sugar). Motile organisms move away from the zone of the stab inoculation.

2.1.9 Spore stain

(Schaeffer and Fulton 1933)

Staining with malachite green detects spores with thickened cell walls. Vegetative cells will stain pink with the safranin stain.

Malachite green spore stain

- 50 g malachite green
- 1 L deionised water

Mix malachite green and water well. Leave overnight and filter to remove undissolved crystals.

Safranin

- 5 g of safranin in 1 L deionised water

1. Prepare smear and heat fix in the usual way.
2. Flood the slide with 0.5% aqueous malachite green and steam for 5 min over a flame. Replenish evaporation loss by adding more stain.
3. Wash well in deionised water and counter-stain with 0.5% aqueous safranin for 1 min.
4. Wash and blot dry.

2.1.10 Classification of isolates based on biochemical tests

Tables 2.1 and 2.2 were adapted by the University of Sydney's Department of Microbiology from Cowan and Steele's manual (Cowan and Steele 1974), and are useful as a quick reference guide for rough identification of bacteria. These biochemical characteristics should be retained by micro-organisms through subculturing and may be used to confirm identity.

Table 2.1 *First-stage tests for gram-negative organisms*

	Genus								
Descriptor	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. Shape	R	S	S	S	R	R	R	R	R
2. Motility	–	–	–	–	+	–	+	+	D
3. Growth (aerobic)	–	–	+	+	+	+	+	+	+
4. Growth (anaerobic)	+	+	–	–	–	–	(1)	+	+
5. Catalase	d	D	+	+	+	+	+	+	+
6. Oxidase	–	X	+	+	+	+	+	+	–
7. Glucose (acid)	D	–	+	–	–	+	+	+	+
8. Carbohydrates (F/O/–)	F/–	–	O	–	–	O	O	F	F
1. *Bacteroides*									
2. *Veillonella*									
3. *Neisseria*									
4. *Branhamella*									
5. *Alcaligenes*									
6. *Flavobacterium*									
7. *Pseudomonas*									
8. *Vibrio*									
9. Enterobacteriaceae									

+ = 85–100% strains positive; – = 0–15% strains positive; d = 16–84% strains positive; D = different reactions given by lower taxa; F = fermentation; O = oxidation; R = rods; S = sphere; X = not known; (1) = some strains use NO3 as an alternative electron acceptor (i.e. they grow anaerobically in presence of NO3)

Source: Adapted from Cowan and Steele (1974).

Table 2.2 *First-stage tests for gram-positive organisms*

	Genus									
Descriptor	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1. Shape	S	S	S	R	R	R	R	R	R	R
2. Acid-fast	–	–	–	–	–	–	–	+	–[a]	–
3. Spores	–	–	–	–	–	+	+	–	–	–
4. Motility	–	–	–[b]	–	–	D	D	–	–	–
5. Growth in air	+	+	+	+	d	+	–	+	+	+
6. Growth anaerobic	–	+	+	+	+	D	+	X	–	–
7. Catalase	+	+	–	+	–	+	–	+	+	+
8. Oxidase	–	–	–	–	–	d	X	–	–	–
9. Glucose	D	+	+	+[c]	+	D	D	+	+	+
10.Carbohydrates (F/O/–)	O/–	F	F	F[c]	F	F/O/–	F/–	O/nt[d]	O[e]	O[e]
1. *Micrococcus*										
2. *Staphylococcus*										
3. *Streptococcus*										
4. *Corynebacterium*										
5. *Lactobacillus*										
6. *Bacillus*										
7. *Clostridium*										
8. *Mycobacterium*										
9. *Nocardia*										
10. *Streptomyces*										

+ = 85–100% strains positive; – = 0–15% strains positive; d = 16–84% strains positive; D = different reactions given by lower taxa; F = fermentation; nt = not tested; O = oxidation; R = rods; S = sphere; X = not known

a Acid-fast to 5% H2SO4.

b Some strains of the streptococci group D are motile.

c Some strains of *Corynebacterium* do not attack glucose.

d As (e) if glucose used.

e Negative or weak reaction due to buffering effect of amino acid products from peptone breakdown.

Source: Adapted from Cowan and Steele (1974).

Identification of BioGro strains

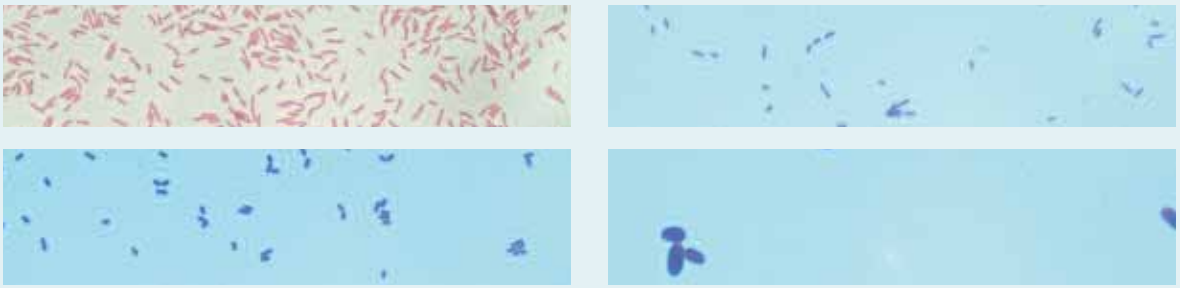
The biofertiliser strains in an earlier formulation of BioGro were identified to family or genus level using results from the tests described above, which are given in the table below. Further biochemical and molecular tests were required to confirm identities to species level.

Nevertheless, each of these tests may be used as a rapid check for strain purity in a broth culture or from a colony with unusual morphology.

The observation of cellular morphology under a light microscope combined with a Gram stain is particularly useful. It is important to note that cell morphology may vary when cells are recovered from different environments—cells from peat cultures or stressful environments may appear smaller than freshly cultured cells. Cell morphology from different conditions needs to be well studied and documented if it is to be a useful means of identification.

Biochemical and physiological characteristics of BioGro strains

Test	***Pseudomonas fluorescens* (1N)**	***Citrobacter freundii* (C3)**	***Klebsiella pneumonia* (4P)**
Gram stain	Negative	Negative	Negative
Oxidase	Positive	Negative	Negative
Catalase	Positive	Positive	Positive
Metabolism (glucose)	Oxidative	Oxidative/fermentative	Oxidative/fermentative
Motility	Motile	Motile	Non-motile



Gram stains of BioGro strains of Pseudomonas fluorescens *(1N) (top left);* Bacillus subtilis *(B9) (top right);* Bacillus amyloliquefaciens *(E19) (bottom left) and* Candida tropicalis *(HY) (bottom right)*

2.2 Nutritional/biochemical identification

For convenience, commercial kits based on a combination of nutritional and biochemical tests are available for the identification of micro-organisms. The API system from BioMerieux (<http://apiweb.biomerieux.com>), designed to give rapid identification, consists of micro-tubes containing dehydrated media to which microbial suspensions are added. The combination of positive and negative reactions for each organism is compared with profiles on a database (Analytical Profile Index) and classification is based on homology with previously identified organisms.

There are several kits for identification including:

- API 20E for bacteria of the family Enterobacteriaceae
- API 20NE for non-fastidious, gram-negative rods except Enterobacteriaceae
- API 50CH for the study of carbohydrate metabolism in micro-organisms
- API 20CAUX for the identification of yeasts.

The system is limited to the species listed on the database.

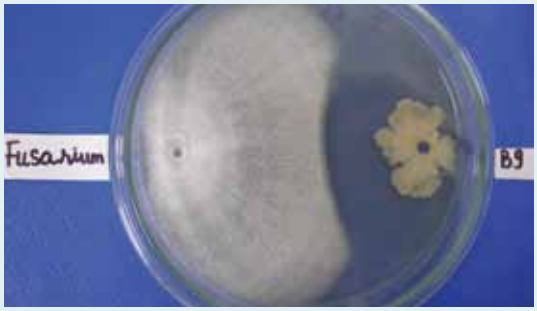
2.2.1 Reagents

Reagents for these commercial methods come with the kits. Pure liquid cultures of test strains are required. Some additional materials may be needed, but these are usually readily available in most microbiology laboratories.

Additional characteristics for strain confirmation

Other specific characteristics of strains can be used to confirm their identity. For example, we have found that the *Bacillus* strain E19 suppresses the growth of *Bradyrhizobium japonicum* and that *Bacillus* strain B9 inhibits growth of *Fusarium.* Most strains will be found to have some distinguishing characteristic that can be used to complete the identification.

Bacillus amyloliquefaciens *E19 suppresses the growth of* Bradyrhizobium japonicum *CB1809 whereas* Bacillus subtilis *B9 does not.*



Antifungal activity of Bacillus subtilis *B9 against* Fusarium

2.3 Genetic identification based on 16s ribosomal DNA sequence

2.3.1 Use of rDNA to identify bacterial strains

Ribosomes provide the site for protein synthesis and are therefore essential organelles in cellular organisms. The RNA sequence encodes the backbone of protein structure and has slowly diverged among microbial species providing a convenient base for molecular-level taxonomy. The coding sequence for the 16S ribosomal RNA (rRNA) gene, a structural component of the small subunit of bacterial ribosomes, can be found on the main DNA of the prokaryotic genomes and consists of about 1,500 nucleotides. Identification is based on comparison of the 16S rDNA gene of individual species to known sequences available in online databases. Steps involve DNA extraction and purification, amplification of 16S rDNA using PCR, sequencing of the nucleotide and comparison of the sequence to others via database search software. References for the application of 16S DNA in phylogenetic studies include Lane et al. 1985 and Weisburg et al. 1991.

Reagents and equipment

- Commercial DNA extraction kit or simple preparation (Qiagen's DNeasy)
- Oligonucleotide synthetising services (for primers)
- PCR machine (or thermocycler)
- PCR mastermix, containing bases, buffers and DNA polymerase
- Microcentrifuges
- DNA gel-electrophoresis apparatus
- Illuminator for visualisation of DNA bands (UV box or a Dark Reader [non-UV])
- DNA sequencing services, if available

Primer design

Design primers using free online or commercial software and allow up to one week for synthesis of the oligonucleotides by a commercial company. See Table 2.3 for commonly used 16s rDNA primers.

Individual nucleotides can be replaced by mixtures of 2, 3 or 4 different nucleotides in equimolar ratios. These are also known as degeneracies or wobbles. Please note that the 3′ nucleotide cannot be a mixed base.

DNA preparation for PCR amplification

The DNA can be extracted by the boiling technique (crude preparation) or by using commercial kits.

Boiling technique

1. Transfer a single colony grown on plate media using a sterile toothpick or pipette tip into a sterile microfuge tube containing 50 mL of sterile DNA-free deionised water.
2. Boil the suspension at 100 °C in a water bath for 10 min to lyse the cells and release their DNA.
3. Centrifuge at 5,000 × g for 1 min and transfer the supernatant (crude DNA extract) to a new sterile microfuge tube.

Extraction of DNA with commercial kit (Qiagen's DNeasy)

Reagents and other accessories for this method come with the kit at the time of purchase.

Pure liquid cultures of test strains are required. Some additional materials may be needed, but these are usually readily available in most microbiology laboratories.

Table 2.3 *Some commonly used universal primers for the partial amplification of 16s rDNA*

Sequence name	Sequence	Number of bases
fD1mod_8_27 left (forward) primer	AGAGTTTGATC**Y**TGG**Y**T**Y**AG	20
16s27f left (forward) primer	AGAGTTTGATCMTGGCTCAG	20
rP_1512_1492 right (reverse) primer	ACGGCTACCTTGTTACGACTT	21
16s1492r right (reverse) primer	TACGG**Y**TACCTTGTTACGACTT	22
515_533 left (forward) primer	GTGCCAGCAGCCGCGGTAA	19
16s530f left (forward) primer	GTGCCAGC**M**GCCGCGG	16
BSR1407_1392 right (reverse) primer	GACGGGCGGTGTGT**R**C	16
16s907r right (reverse) primer	CCGTCAATTC**M**TTT**R**AGTTT	20

The International Union of Biochemistry single letter codes for mixed nucleotides are: A,C = M; A,G = R; A,T = W; C,G = S; C,T = Y; G,T = K; A,G,C = V; A,C,T = H; A,G,T = D; C,G,T = B; A,G,C,T = N

Amplification of target 16s rDNA sequence by PCR

A thermocycler, where different temperatures allow denaturation of DNA, annealing of primers and extension of new DNA, is used for PCR. A light cycler is used for real-time PCR along with fluorescent dyes that bind to DNA. DNA amplification is monitored using laser pulses that detect increasing levels of fluorescence over time (Figure 2.1).

1. Mix 5 mL of DNA extract, 45 mL DNA-free water, 10 mL primer 1 (forward primer: GTGCCAGC**M**GCCGCGG) (1 pmol/µL), 10 mL primer 3 (reverse primer: GACGGGCGGTGTGT**R**C) (1 pmol/µL (where M = AC and R = AG) and 30 mL Taq mastermix in a sterile PCR tube or plate.
2. Use the following temperature program:
   a. Initial denaturation temperature of 94 °C for 3 min.
   b. 38 cycles of
      – denaturation at 94 °C for 30 s
      – annealing at 55 °C for 30 s
      – extension at 72 °C for 1 min.
   c. Final extension at 72 °C for 7 min.
   d. Hold at 4 °C.

Note: As with all PCR reactions, the above conditions can be optimised to suit the requirements. Also, higher-quality sequences can be obtained by purifying the amplification products using commercial kits such as Qiagen's QIAquick kit.

Figure 2.1 *LightCycler used for real-time PCR is interfaced with computer software to record amplification of DNA over time*

Confirmation of PCR products using gel-electrophoresis

1. Prepare a 1.1% agarose gel in TAE buffer (40 mM Tris base, 0.114% (v/v) glacial acetic acid, 2 mM EDTA, pH 8) containing 0.5 µg/mL ethidium bromide. (Warning: Ethidium bromide is highly carcinogenic; exercise caution in handling and use only in a dedicated laboratory space. Alternatively, use SYBR green as the DNA staining reagent.)
2. Mix the loading dye (4 µL) and PCR product or marker (10 µL) samples and run the gel at 100 V for 45 min to 1 h at constant voltage.
3. After completion of the electrophoresis, visualise the DNA with a UV light (if using ethidium bromide) or blue light (if using SYBR green and Dark Reader technology), and photograph it using a gel documentation system.
4. Determine the concentration of the PCR product in solution using a spectrophotometer (260 nm), or with gel electrophoresis, based on the product's band intensity compared to a standard DNA marker set run in parallel.

Sample preparation for sequencing

Follow the recommendation of the sequencing company.

In brief:

1. Prepare two separate samples (in tubes or in the PCR plate) for sequencing as follows: purified PCR products, DNA-free water, and the forward or reverse primer used in the PCR.
2. Send/deliver the 16s rDNA PCR product for sequencing.

Data alignment and bacterial identification using the National Center for Biotechnology Information (NCBI, United States of America)

1. Merge and confirm sequences initiated by the forward and reverse primer pairs using free online or commercial tools like Geneious (Drummond et al. 2010, *(*<www.geneious.com>).
2. Match the generated (consensus) sequences to the NCBI database and determine the identity of the bacterial strains up to species or strain level (e.g. Figure 2.2).

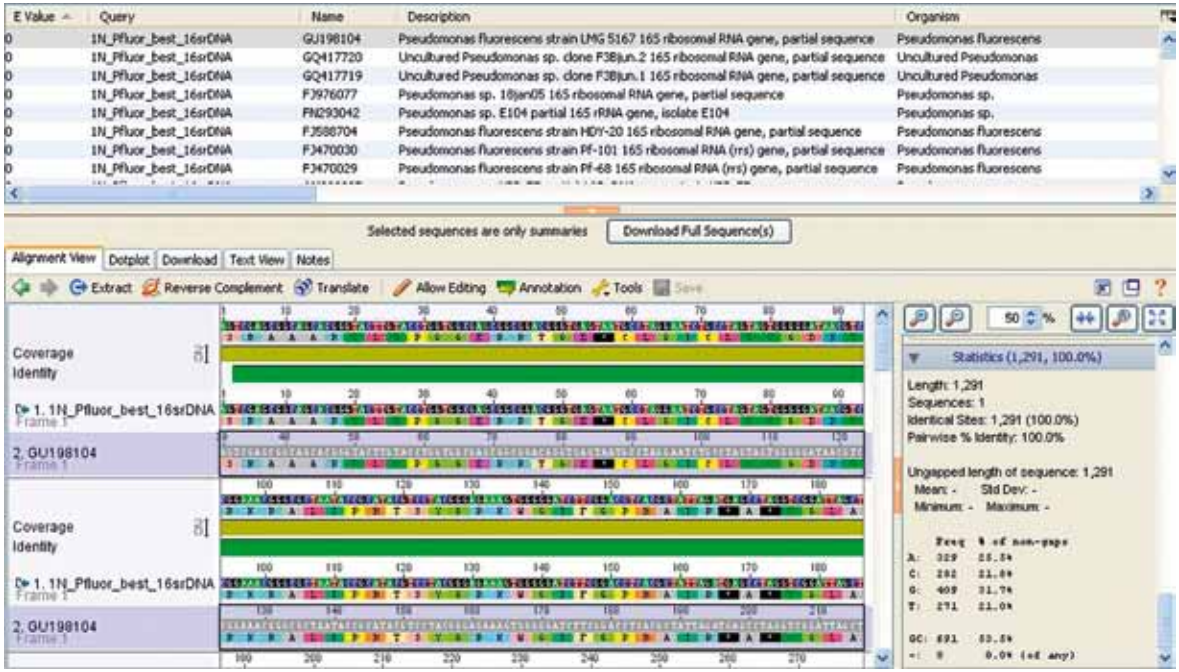


Figure 2.2 *Biofertiliser strain 1N identified as* Pseudomonas fluorescens*, using a Blast N search of the NCBI database*

03

Enumeration of inoculant strains

The enumeration and identification of selected organisms in biofertilisers is crucial to predict their effectiveness. Primarily, these methods are needed to validate the identity and quantity of specific plant growth-promoting micro-organisms in the inoculant, so that farmers can be certain the product is of sufficient quality and can test the product with confidence under their local environmental conditions. Secondly, counting methods are necessary for research purposes to optimise the efficacy of biofertiliser inoculants. Of particular importance is the survival of strains in the inoculant over time to determine shelf life, or enumeration of plant growth-promoting cell numbers after application to follow their persistence in the field.

The main problem regarding counting of plant growth-promoting inoculants is their variability. No single medium is suitable to selectively cultivate the range of microbial isolates identified as potential plant growth-promoting micro-organisms. These isolates have different plant growth-promoting traits, plant specificities, nutritional requirements, growth rates, preferences for particular environmental conditions like pH and survival capabilities. Although all of these differences are reflected in their genetic composition, their interaction with the environment and other organisms means that their actual behaviour will change from site to site.

Thus, enumeration methods need to be specific (for either a particular plant growth-promoting *organism* or *function*), sensitive enough to detect low population levels and robust enough to provide consistent results under different laboratory and field conditions. The methods should also be rapid, inexpensive and relatively easy to use.

Enumeration methods are both qualitative, relating to the specificity of the method being used, and quantitative, relating to the sensitivity of the method required. Note that the quality standard may either be species specific (e.g. 'This product contains 10^7 cfu/g of *Azospirillum brasilense*') or function specific (e.g. 'This product contains 10^7 most probable number (MPN)/g of wheat-associative nitrogen-fixing micro-organisms').

There are many enumeration methods that can be used, each with their own advantages and limitations (Table 3.1). Most importantly, methods should be specific, quantitative and indicate viability. Many of the tests developed to date only meet two of these criteria; there are few that satisfy all three.

3.1 Strip plate counting and use of selective media

Routine counting of microbial strains is based on serial dilution of liquid culture followed by growth on media. Methods such as spread plate counting of colonies use significant amounts of media and consumables, such as cell culture dishes, as only one dilution can be spread on each plate. Miles and Misra counts and strip plating are methods developed to reduce the number of plates needed and time required thereby reducing costs.

Table 3.1 *Characteristics of counting methods*

Enumeration method	Capital cost	Variable cost	Time to develop	Assay time	Specificity	Factors affecting accuracy in counting	Measure of viability
Plate count	Low	Low	Fast	Slow	Low	Colony growth[a]	Yes
Selective plate count	Low	Low	Fast	Slow	Medium	Colony growth[a]	Yes
Multiple-tube fermentation	Low	Low	Fast	Slow	Medium	Dilution factor and tube number	Yes
Indirect ELISA	Medium	Low	Slow	Fast	High	Sensitivity and calibration	Includes dead cells
Immunoblotting	Medium	Medium	Slow	Slow	High	Colony growth[a]	Yes
Sandwich ELISA	Medium	Low	Slow	Fast	High	Sensitivity and calibration	Includes dead cells
Quantitative real-time PCR	High	High	Medium	Fast	Very high	Sensitivity and calibration	Includes dead cells (DNA)

ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction

a Colony growth may be restricted by competition or inhibition by colonies of contaminating organisms. The extent of colony competition is determined by the relative level of purity at the most suitable dilution level. If the number of contaminants is ten-fold lower than the number of a biofertiliser strain, then unrestricted colony growth should be possible. Accuracy of colony counting from pure cultures is affected by high numbers where growth may be restricted or low numbers where statistical variation is magnified. The same considerations of accuracy apply for mixed cultures. Similarly, highly selective media may restrict colonies from cells that have been stressed (e.g. desiccated) to a certain extent.

Serial dilutions are still required, but volumes of each dilution can be reduced. In the case of Miles and Misra counts, small volumes of each dilution are placed as drops on the surface of plates, usually divided into segments, and colony growth occurs where the drops have been placed (6–8 dilutions/plate). Alternatively, the drops are run down lanes in the same plate allowing the volume to be spread over a larger surface area (up to 5 dilutions/plate; Figure 3.1). These methods are useful for enumerating pure cultures or species/strains with dry colony morphologies. Inaccuracies may occur if cultures are not pure or if colonies are gummy or spreading as there is less area for separation of colonies compared with spreading cell suspensions over the entire agar surface. Errors are also likely to be larger because of the small volumes employed; however, this can be offset by the larger number of replications that are possible.

3.1.1 Reagents

- 3 solid nutrient agar (NA) plates per strain
- 50 mL of 0.85% sterile NaCl

3.1.2 Strip plating

1. Prepare solid media (plates) and tubes containing sterile NaCl (0.9 mL) for serial dilutions.
2. Serially dilute cultures by transferring 0.1 mL to 0.9 mL NaCl and repeating until adequate dilution has been achieved (for overnight broth cultures, dilution to 10^{-7} is usually sufficient).
3. Using a fine marker, draw five parallel, vertical lines on each plate, about 1 cm apart to make four lanes. Label each lane with the dilution to be plated.
4. Starting at the highest dilution, withdraw 10 µl of liquid. Hold the plate on a 45 degree angle, with the lanes running vertically, and expel the liquid at the top of the lane.
5. Run the liquid down the plate, keeping it within the lane. Do not let the liquid touch the edge of the plate at the end of the lane. Repeat for each dilution.
   Note: Control of running liquid over agar may be affected by dryness of the agar surface. This should ideally be optimised before using for routine analysis.
6. Incubate until colonies are visible, taking care not to allow overgrowth of colonies.

3.1.3 Plate counting using selective antibiotic media

This protocol is described using nutrient agar as the base media but any media can be used as long as it supports the growth of the organisms in question.

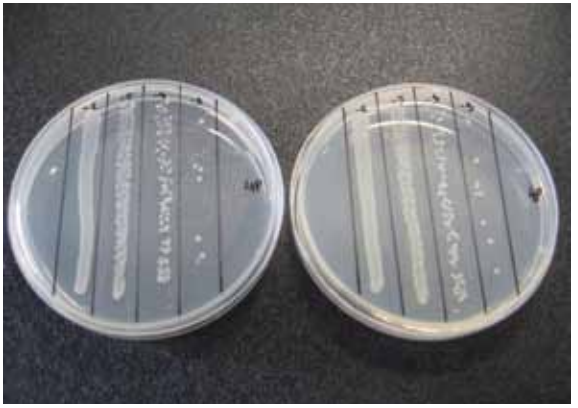


Figure 3.1 *Strip plating of BioGro strains showing colony growth within lanes marked on the plate*

Reagents and media

- Nutrient broth
  - 8 g of nutrient broth powder (5 g peptone and 3 g beef extract or lab lemco)
  - 1 L of deionised water
  - for solid medium add agar (15 g/L)
- Antibiotics (added according to specific strain sensitivities—see Table 3.2)
  - tetracycline 10 mg/L
  - vancomycin 10 mg/L
  - cycloheximide 100 mg/L

Filter-sterilised antibiotics should be added to sterilised media after cooling to about 60 °C. However, antibiotic solutions may not need to be filter sterilised if prepared using ethanol.

Table 3.2 *Growth on antibiotic media*

Strains	Vancomycin	Tetracycline	Cycloheximide	Vancomycin + cycloheximide
1N	Growth	No	Growth	Growth
HY	Growth	Growth	No	No
B9	No	No	Growth	No
E19	No	No	Growth	No

3.1.4 Protocol for counting micro-organisms in peat using antibiotic media

To determine the number of viable cells in peat cultures, aseptically weigh 10 g of each and make volume up to 100 mL using phosphate buffered saline (PBS) or sterile deionised water (volumes for initial dilution will vary according to moisture characteristics of peat and should be calibrated beforehand). Shake for 15 min on a wrist-action shaker and prepare serial dilutions by transferring 0.1 mL to 0.9 mL sterile PBS (pH 7.0) until a dilution of 10^{-6} is obtained. Spread 0.1 mL of 10^{-4}, 10^{-5} and 10^{-6} dilutions on NA plates using a sterile spreader. Alternatively use strip-plate, or Miles and Misra techniques.

Media for selection of BioGro strains is as follows:

- For the determination of 1N, plate onto NA modified with cycloheximide and vancomycin.
- For the determination of HY, plate onto NA modified with tetracycline.
- For the determination of B9 and E19, plate onto NA plates with cycloheximide.
  Note: 1N will still grow on this media. B9 and E19 will need to be determined using colony morphology.

Incubate all plates at 30 °C for 24–48 h. When counting colonies, compare to referenced pure culture colonies to visually confirm their identity. Further conformational tests may be performed on randomly selected colonies.

3.2 Multiple-tube fermentation using differential media

One approach to counting a specific strain from a mixture of other strains is to use selective or differential media. Selective media allows only the strain of interest to grow (e.g. media containing antibiotics). Differential media allows differentiation of strains based on their ability to carry out biochemical reactions that are unique within a mixed culture. In this case, a suitable medium would include a specific carbohydrate that is fermented by only one strain in a mixed culture. The acid produced changes the colour of the media from red to yellow via the indicator phenol red. All organisms may grow in this media, but only organisms that ferment the specific carbohydrate produce the colour change. Media that allows growth or detects a biochemical reaction of a particular organism within an ecological group can be used in multiple-tube fermentation techniques (Alexander 1965) to estimate the most probable number of specific physiological groups in an environment.

3.2.1 Most probable number method

The MPN method estimates viable cell numbers based on probability and the presumption that one cell will produce a positive response in a tube. The numbers are usually estimated using published tables or software such as MPNes (Most Probable Number Enumeration System) produced by NifTAL. The mathematical basis of MPN table construction has been published in several papers (see Gonzalez 1996). The probability of the number of organisms in a known volume is based on Poisson theory whereby the probability of having a negative tube is equal to $e^{-\nu\delta}$ (where ν is the volume and δ is the density of cells). Accuracy of the method increases with the number of tubes inoculated at each dilution, as distribution of suspended cells are typically non-homogeneous, or by reducing the base of the dilution ratio. The inaccuracy of the MPN method is often reflected in large fiducial limits.

Reagents and equipment

- Saline (0.85% sterile NaCl)
- Differential media
  - 890 mL phosphate buffer, pH 7.0
  - 2 g/L ammonium sulfate
  - 0.5 g/L yeast extract
  - 1 g/L tryptone
  - 0.18 g/L phenol red
  - 100 mL/L carbohydrate (concentrated)
  - 10 mL trace elements
- Trace elements for differential media
  - 0.2 g/L sodium molybdate
  - 0.235 g/L manganese sulfate
  - 0.28 g/L boric acid
  - 0.008 g/L copper sulfate
  - 0.024 g/L zinc sulfate
- Phosphate buffer for differential media
  - 1.21 g/L dipotassium hydrogen phosphate
  - 0.34 g/L potassium dihydrogen phosphate
- Differential carbohydrate solution. Make a concentrated 5% stock solution of the differential carbohydrate solution. Filter sterilise.

Most probable number dilution series

1. Prepare serial dilutions of the test sample (up to 10^{-8} for pure cultures of growth media, up to 10^{-6} for soil samples) starting with 10 g of soil or peat thoroughly mixed in 100 mL of sterile phosphate buffer (pH 7.0).
2. Prepare microfuge tubes containing 0.9 mL sterile differential media for each dilution in triplicate (thus, for pure cultures you will need 27 tubes). Inoculate 3 tubes with 100 µL of each dilution.
3. Incubate tubes for 24 h at 30 °C.
4. Record the number of positives for each dilution. A positive result is a change from red to yellow media (Figure 3.2).
5. Calculate the MPN using published tables or software.

3.3 Polyclonal antibody production and purification

Biofertiliser strains are cultured, heat-killed, mixed with an adjuvant and injected into rabbits. Proteins and/or exopolymers from the strain act as antigens, initiating an immune response in the rabbit and the production of polyclonal antibodies. Polyclonal antibodies are a mixture of large immunoglobulin molecules that recognise and bind to specific chemical residues. These antibodies can then be purified and used for detection and quantification of the specific strain.

In this example the results would be recorded as follows:

Dilution level	Number of positive tubes
10^{-1}	3
10^{-2}	3
10^{-3}	3
10^{-4}	3
10^{-5}	2
10^{-6}	1
10^{-7}	0
10^{-8}	0
10^{-9}	0
10^{-10}	0

Figure 3.2 *Multiple-tube fermentation is a useful way to determine the most probable number (MPN) of micro-organisms; results are recorded as positive (yellow) or negative (red) and the MPN is calculated using published tables or software.*

3.3.1 Reagents and equipment

- 3 NA plates per strain
- 50 mL sterile NaCl (0.85%)
- 10 mL Freund's adjuvant
- Protein A sepharose column
- Column buffer (10 mM phosphate-buffered NaCl, pH 8.0), 1 L
  - 9.0 g NaCl
  - 0.5 g NaN_3
  - 1.35 g Na_2HPO_4
  - 0.075 g $NaH_2PO_4.H_2O$
- Elution buffer (0.1 M sodium citrate buffer, pH 4.0), 500 mL
  - 6.4 g tri-sodium citrate
  - 6.18 g citric acid
- Rinse buffer (0.1 M citric acid, pH 1.9), 500 mL
  - 10.5 g citric acid
- 1 M tris base, pH 11.0

3.3.2 Inoculum preparation

1. Culture pure microbial strains on agar plates for 24–48 h.
   Note: Ideally, defined media should be used as natural extracts in media may be a source of antigens.
2. Wash cells from grown plates with 3 aliquots of 5 mL of sterile saline (0.85% NaCl); detach cells using a sterile glass spreader and transfer suspended culture to sterile centrifuge tubes.
3. Centrifuge, remove supernatant and wash two times with 5–10 mL of sterile saline, centrifuging and vortexing after each. After the second wash, resuspend bacteria in sterile saline to obtain approximately 10^9 cfu/mL (this can be quickly checked by the McFarland estimation).
4. Refrigerate until used for inoculation.
5. Depending on the pathogenicity of the microbial strain, cell suspensions can be heat-killed by placing vials containing the suspension in a boiling water bath for 1 h.

Versatility of the most probable number technique

The most probable number (MPN) technique is not restricted to use in combination with differential carbohydrate media. A number of other physiological observations or reactions can be used to differentiate and then enumerate the functional microbial groups of interest. These can include hydrocarbon degraders (Johnson and Henriksen 2009), nitrifiers (Feray et al. 1999), iron-reducers and iron-oxidisers (Weiss et al. 2003), and methanotrophs (Escoffier et al. 1997). With respect to plant growth promotion, one of the best and most widely applied examples of an MPN method is for the enumeration of nitrogen-fixing rhizobia (Brockwell 1963). In this technique, serial dilutions of the sample of interest are inoculated into plant infection tubes and a positive test is scored when nodule formation is observed. An alternative to this is the inoculation of serial dilutions into N-free media, with positive tubes scored by an acetylene reduction assay (Okon et al. 1977).

In fact, most functional tests can be adapted for MPN enumeration. For example, organic P-mobilising bacteria can be enumerated by inoculating serial dilutions into a growth medium containing phytate as a sole source of P. After incubation overnight, Wade's reagent (Makkar et al. 2007) is added to each tube. If phytate is still present, it will bind the iron in Wade's reagent, and the media will stay colourless. If the phytate has been used by any of the micro-organisms present in the sample, the iron in Wade's reagent will remain bound by sulfosalicylic acid, and the tube will turn pink–purple and can be scored as positive. This can be seen on the right in the tubes inoculated with strain HY.

Pink–purple tubes indicate phytase activity in yeast strain HY

3.3.3 Antibody production

1. Each strain should be inoculated into at least two rabbits.
   Note: Rabbits should be cared for under animal welfare guidelines and injections made by trained personnel.
2. Emulsify 1 mL of the cell suspension (10^9 cfu/mL) with 1 mL of Freund's incomplete adjuvant by drawing both solutions into a syringe (20 gauge needle) and rapidly ejecting into a vial.
3. Inject each rabbits once a week for 4 weeks with 1 mL of emulsified cell suspension: one rabbit should be injected intramuscularly and the other subcutaneously.
4. Test-bleed the rabbits after 5 weeks and determine the titre by micro- or tube-agglutination.
5. Continue injections until a titre of at least 1:2,560 or greater is obtained.
6. Once a positive agglutination test result has been obtained (≥ 1:2,560), use an ELISA test to confirm sensitivity and specificity.

3.3.4 Agglutination testing

1. Dilute a volume of the heat-killed antigenic strain to make 5 mL of antigen solution that is representative of 10^7 cfu/mL (for bacteria) or 10^6 cfu/mL (for yeast).
2. Set up a two-fold dilution series of the antiserum from 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, 1:12,800 and 1:25,600 by diluting each aliquot with the same volume. This can be done as 0.2 mL volumes in microfuge tubes, small glass tubes or 96 well plates. There should be 9 tubes containing antisera at different dilutions plus a control tube with no antisera.
3. Add 0.2 mL of antigen suspension to each antisera dilution. Agglutination should be observable as a precipitation of the antigen–antisera complex. The titre of the antiserum is defined as the reciprocal of the highest dilution causing agglutination that can be seen by the naked eye. For example, if the highest dilution causing agglutination is 1/600, the antiserum titre is 600.
4. Note that agglutination is dependent on both the antiserum and antigen concentration. The tube agglutination can be repeated with a different concentration of antigen to validate results of the first test. A titre greater than 3,200 is deemed as a sensitive antiserum.

3.3.5 Antiserum purification (immunoglobulin G [IgG] fraction)

1. Dilute the blood serum with an equal volume of serum diluent (column buffer with 25 mM e-caproic acid).
2. Allow the column, column buffer, elution buffer and rinse buffer to come to room temperature.
3. Turn on the UV detector at least 20 min before starting the purification. The detector should be set to read the absorbance at 280 nm.
4. Wash the column with column buffer until it reaches pH 8.0. Throughout the purification procedure, do not let the column become dry between buffer additions.
5. Set the baseline absorbance on the chart recorder to zero while the column buffer is running (pump at 1 mL/min). Wait until the baseline is stable before continuing.

6. Load the diluted serum onto the column, followed immediately by column buffer. Generally speaking, the amount of column buffer required in this section will be twice the volume of the diluted serum. However, column buffer should be added continuously in smaller volumes; see steps 7 and 8.
7. Once the baseline starts to rise, begin collecting fraction #1 until the absorbance returns to the baseline (i.e. when the absorbance returns to zero).
8. When the absorbance has returned to the baseline, add elution buffer. Collect fraction #2 as the absorbance rises. This is the antibody fraction. When the absorbance starts to go down, change to column buffer and continue to collect until absorbance returns to the baseline.
9. Immediately neutralise the eluate with 170 µL of 1 M Tris base (pH 11.0) per mL of eluate (fraction #2) collected.
10. Wash the column with 10 mL of rinse buffer or until the absorbance returns to the baseline and collect fraction #3.
11. Wash the column with column buffer until the pH returns to 8.0.
12. The column can now be used again or stored at 4 °C.
13. For storage longer than 5 days, pump 10% ethanol through the column before storing at 4 °C.
14. Pipette the antibody fraction inside a piece of dialysis tubing and place in a beaker containing PBS after sealing both ends of the tubing. Because IgG monomers are approximately 150 kDa, the dialysis tubing should have a molecular weight cut-off of 20 kDa or less, with a diameter between 5–10 mm. Place the beaker in a cold room on a magnetic stirrer and leave overnight. The next day, change the PBS and leave overnight. On day three, change the PBS again and leave overnight.
15. Absorbance of the antibody fraction should be measured using a spectrophotometer and a quartz cuvette. Dilute the antibody fraction before reading its absorbance (1 in 20 is usually sufficient).
16. Protein concentration (mg/mL) = (1.55 × Abs 280 nm) – (0.76 × Abs 260 nm)

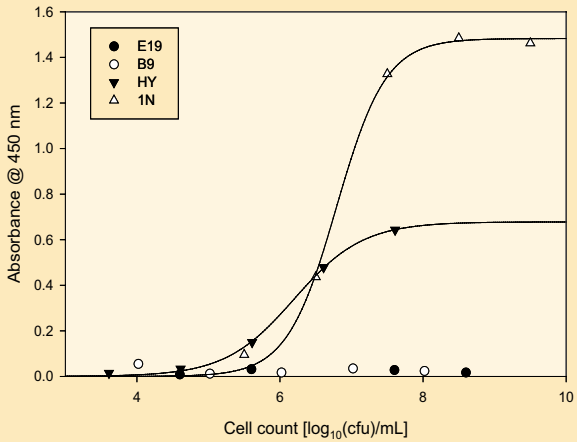
3.4 Indirect enzyme-linked immunosorbent assays

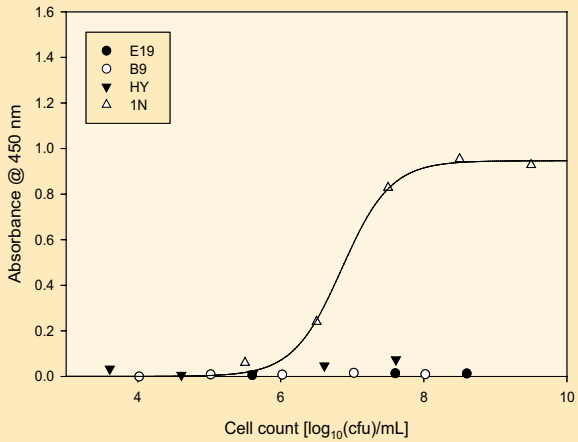
Polyclonal antibodies produced against a microbial inoculum will exhibit a different sensitivity and specificity from strain to strain and animal to animal. ELISAs can quantitatively measure the sensitivity of an antibody preparation binding to a certain microbial strain and also determine if there are cross-reactions with different microbial strains. A cell suspension is coated onto a 96-microwell plate, after which the primary antibody is bound to the cells (Figure 3.3). After washing to remove unbound primary antibody, a secondary, anti-rabbit, enzyme-conjugated antibody is added to bind to the primary antibody. This enzyme conjugate can then react with a substrate to produce a colour, with the intensity of the colour directly related to the initial number of microbial cells present in each well.

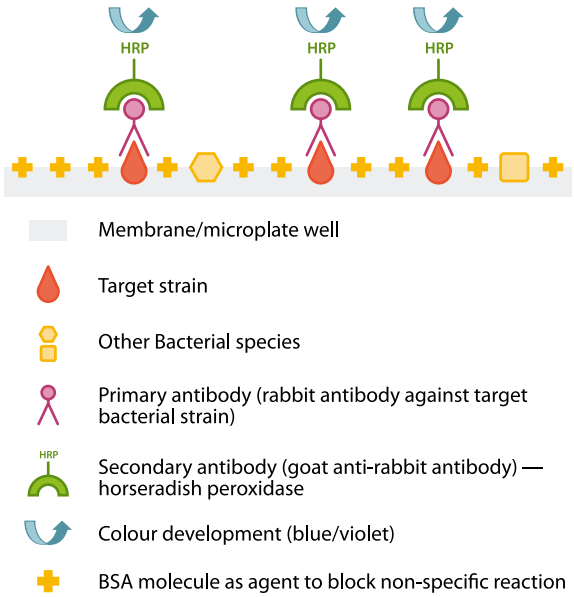
Removal of cross-reactivity from antisera

The majority of the unpurified antisera produced for the strains comprising BioGro cross-reacted against the HY yeast antigen. Although the exact antigenic residues could not be determined, the cross-reactivity was removed after purification on protein A sepharose, suggesting that the cross-reacting proteins were not immunoglobulin G (IgG) components.

Reaction of unpurified 1N antisera (left) and purified 1N antisera (right) against 1N, HY, B9 and E19 heat-killed cell antigen







BSA = bovine serum albumin; HRP = horseradish peroxidase

Figure 3.3 *Indirect immunoassays, as used in ELISA and immunoblotting, involves a primary antibody specific for the strain of interest and a secondary anti-rabbit HRP-conjugated antibody*

3.4.1 Reagents

- Carbonate buffer—dissolve 1.6 g Na_2CO_3 and 2.9 g $NaHCO_3$ in 1L of deionised water; pH 9.6
- PBS—dissolve 17.4 g NaCl, 40 mL phosphate buffer (6.177 g Na_2HPO_4 and 1.014 g NaH_2PO_4 in 100 mL of deionised water) and make up to 2 L with deionised water; pH 7.4
- Blocking solution—PBS, 0.2% polysorbate 20 (commonly known as Tween 20) and 5% skim milk powder
- Washing solution—PBS + 0.2% polysorbate 20
- Substrate—0.1 M sodium acetate, 0.25% (w/v) ß cyclodextrin, and 43 mg/100 mL urea H_2O_2 or 5 mL 30% H_2O_2. Adjust to pH 5.0 with acetic acid or citric acid. Be sure to check the pH before using.
- Chromogen—10 mg/mL TMB (3,3',5,5' tetramethylbenzidine) dissolved in DMSO or DMF. Store in a glass vial in the dark at room temperature.
- Stop solution—10% v/v H_2SO_4. Caution: This is a strong acid; please refer to the correct material safety data sheet to avoid corrosive burns.

3.4.2 Preparation of antigen-coated plates

1. Make up 30 mL of carbonate buffer for one plate. Pipette 2 mL into small glass or plastic tubes; use one tube per bacteria strain. Into each tube mix 3 loopfuls of the bacteria to be tested and vortex thoroughly. Also mix 100 mg of protein into one tube as a blank.
2. Pipette 200 µL of mixed solution into each well of a 96-well plate. Record the well treatments on a plate diagram. Incubate the plates overnight at room temperature or for 2 h at 37 °C.

3.4.3 Indirect immunoassay

1. Empty the carbonate buffer from the 96-well plates and add 200 µL of freshly prepared blocking solution to each well. Incubate for 1 h, with agitation if possible.
2. Incubate the plates again for 1h (or more) at 37 °C in 200 µL of blocking solution containing antibody at the required dilution. For testing of new antisera, 1:200 and 1:2,000 dilutions are recommended as a starting point. Determine the total volume required first (e.g. if 10 wells are required of a 1:2,000 dilution, mix 5 µL antiserum

with 995 µL blocking solution, vortex, then mix 100 µL of this solution with 900 µL of blocking solution).

3. Wash plates twice in a bath of washing solution.
4. Incubate each well with 200 µL of anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibody solution (10 µL of HRP-conjugate antibody in 9.99 mL of blocking solution) for 1–2 h at 37 °C.
5. Carry out two successive 5 min incubations at 37 °C in washing solution and one in PBS.
6. Add 200 µL HRP substrate (per 10 ml: 0.5 mL of chromogen and 9.5 mL of substrate per plate) and wait until the yellow colour develops (usually 1–5 min).
7. Add 10 µL of 1 M H_2SO_4 stop solution to each well and read the absorbance of the colour at 450 nm after 15 min.

3.5 Immunoblotting

Immunoblotting follows the same theoretical steps as an indirect ELISA, but rather than transferring microbial suspensions to wells, microbial colonies from serial dilutions are directly imprinted onto a membrane for subsequent ELISA from a spread plate. In this manner, spread-plate colonies can be directly identified and counted non-destructively, and the original plate can be re-sampled if further tests are necessary.

3.5.1 Reagents

Reagents used are as in ELISA protocols above. Note that immunoblotting requires larger volumes of reagents than ELISAs.

3.5.2 Sample preparation

1. Dilution series are necessary. For liquid culture, these can be done as normal.
2. For soil samples, weigh 1 g of soil and put into 100 mL of sterile deionised water. Shake the suspension for 1 h. If possible, use a rocking/gyratory shaker, otherwise use an orbital shaker.
3. Afterwards, spread 100 µL of each dilution (between 10^{-3} and 10^{-7}) onto solid growth media favourable to growth of the strain of interest.
4. Incubate plates at 30 °C overnight or longer, until colonies have grown.
5. For the immunoblot, choose the best agar plates (e.g. ones where colonies are not overcrowded or numbers too low. Ideally colony numbers should be between 30 and 300 depending on colony size and morphology) (Figure 3.4).

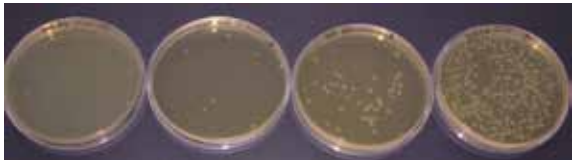


Figure 3.4 *Number of colonies on plate after spreading 100 µL aliquots from a dilution series. Plates selected for calculation of numbers should be high enough to decrease error but not so high that plates are overcrowded. Selection of the colony number will depend on colony size and morphology.*

3.5.3 Colony immunoblotting

1. To transfer the colonies, cover the plate with round nitrocellulose filters (0.45 µm) for a minimum of 30 min at room temperature, making sure that the nitrocellulose filter is in complete contact with the surface of the media to ensure all colonies are transferred. Mark the cell culture dish and nitrocellulose filter at a point so that they can be re-orientated after the staining procedure.
2. Place nitrocellulose membranes on paper tissue (colonies facing up, not touching the tissue) and 'fix' the colonies to the membrane by incubating for 30 min at 80 °C. Heat-fixed membranes can be stored at room temperature until use for colony immunoblotting.

Do not allow the membranes to dry out between steps 3 and 8.

3. Incubate the filters (in cell culture dishes) with agitation (approx. 85 rpm) for 1 h at 37 °C in a freshly prepared blocking solution. About 4 mL of blocking solution is recommended when using cell culture dishes (90 mm diameter). If possible, use a rocking/gyratory shaker, otherwise use an orbital shaker.
4. Incubate the membranes for at least 1 h at 37 °C in 4 mL of blocking solution containing 5 µg/mL of antibody (20 µL of antibody and 4 mL of blocking solution is enough for one plate). This can be modified depending on the antibody-strain sensitivity, as determined by ELISA.
5. Incubate the membranes three times for 5 min in 4 mL of blocking solution at 37 °C.
6. Incubate the membranes in 4 mL of HRP-conjugated secondary antibody for 1–2 h (use 3 µL of secondary antibody and 3.997 mL of blocking solution for one plate).
7. Incubate the membranes twice for 5 min in washing solution and once in PBS, both at 37 °C.
8. Add the HRP substrate (for 3 ml, use 150 µL of chromogen and 2.850 mL of substrate per plate) and wait until colour develops (usually between 1 to 5 min).

Perform step 9 in a fume hood, or with adequate eye, skin and lung protection.

9. Spray sulfuric acid (10%) on the membrane using a perfume spray bottle, without removing the HRP substrate. Shake the membranes so that the HRP substrate–sulfuric acid mixes onto the whole filter.
10. Wait a few seconds until all spots change colour. Take the membrane from the dish and dry between two layers of paper towel.
11. Briefly rinse the membrane in a cell culture dish containing deionised water. Dry the membranes again in between paper towels. A blue colour indicates positive detection of a specific micro-organism (Figure 3.5).

Figure 3.5 *Purple spots indicate positive detection of target micro-organisms; negative colonies can be identified by the absence of purple colour, as indicated by the superimposed circles.*

3.6 Sandwich enzyme-linked immunosorbent assays

If purified antibodies have a high specificity and sensitivity, they can be directly conjugated to a detection enzyme, such as HRP, to reduce analysis time and costs by avoiding the use of a secondary antibody. Although it is similar to the conventional indirect ELISA, the sandwich ELISA takes advantage of the enzyme-conjugated primary antibody, allowing for a more specific and routine test method. Antibodies are coated onto the surface of wells and microbe test solutions are added. After incubation, the wells are washed with a wash buffer and a primary antibody-conjugate is added. Following a second incubation and wash, substrate is added, which develops into a blue colour, and turns yellow after the addition of stop solution. Results are read using a spectrophotometer (strip or plate reader) and comparing the absorbance of the samples to the absorbance of negative and positive controls.

3.6.1 Reagents

Antibody–enzyme conjugation

- Dialysis membrane (molecular weight cut-off less than 20 kDa)
- Purified HRP (available from Sigma or Dako in 10–100 mg batches)
- Sodium periodate ($NaIO_4$) (Sigma-Aldrich, catalogue no. S1878)
- 1 mM citrate buffer, pH 4.5
- 1 M Na_2CO_3, pH 9.5
- 2 M ethanolamine, pH 9.6

Sandwich ELISA

- Pre-prepared antibody-coated strips (12 wells/strip) or 96-well plates
  Note: Antibody-coated plates can be prepared similarly to the coating of plates with cell cultures in the indirect ELISA described in Section 3.4.1. Concentrated antibody stock should be diluted at least 1:1,000 in carbonate buffer (pH 9.5), added to wells and incubated at 37 °C for 2 h.
- Positive and negative control samples. Positive control samples should contain cells of the biofertiliser strain under consideration at a concentration deemed to be functional (e.g. 5×10^6 cfu per mL). Negative control samples should contain cells of a non-target bacteria that is non-reactive to the IgG-HRP conjugate, at a similar concentration to the positive control.
- Enzyme–antibody conjugates. Concentrated stock requiring dilution before use.
- Substrate/TMB solution. PBS; wash solution; stop solution. Prepared as in the indirect ELISA in Section 3.1.4.

3.6.2 Antibody preparation (day 1)

1. Dialyse the purified primary antibody in PBS overnight at 4 °C to remove NaN_3, which is a known inhibitor of HRP activity. The following day, change the PBS twice, with a 2 h incubation each time at 4 °C to ensure complete removal of NaN_3.

3.6.3 Enzyme conjugation (day 2)

1. Dissolve 5 mg of HRP in 1.0 mL of deionsed water.
2. Add 0.2 mL (i.e. 1/5 volume) of 0.025 M sodium periodate (e.g. 21.4 mg $NaIO_4$ in 4 mL of deionised water) to the solution from step 1. The colour of the HRP solution will immediately change from gold to green.
3. Mix gently for 20 min at room temperature on a mixing wheel (approx. 16 rpm).
4. To remove unbound $NaIO_4$, use gel filtration columns (e.g. Pharmacia PD-10, catalogue no. 17-0851-01) that have been equilibrated with 25 mL of 1 mM citrate buffer, pH 4.5.

   Add the solution from step 3 to the PD-10 column (maximum sample volume is 2.5 mL) and elute with 1 mM citrate buffer, pH 4.5. Collect the gold-coloured fractions, which are the HRP aldehydes. Protein concentrations can be done using a Bradford protein assay. When measuring the absorbance, read at 402 nm, not 280 nm. HRP (1 mg/mL) gives an absorbance reading of 2.25. The expected recovery from the columns is 65–75%.
5. Assuming the antibody IgG is approximately 2 mg/mL and the HRP is approximately 1 mg/mL, add equal volumes of the dialysed antibody IgG and the HRP–aldehyde solution. Either or both of the individual solutions can be diluted beforehand, as long as the final ratio of approximately 2 mg antibody to 1 mg HRP–aldehyde is kept. However, try to keep the dilution to a minimum in order to maintain sensitivity of the final IgG-HRP conjugate.
6. Add 1 M Na_2CO_3, pH 9.5 (approx. 50 µL of 1 M Na_2CO_3 per 2–3 mL of coupled solution) and check the pH value. Continue adding the Na_2CO_3 buffer to the solution until the pH reaches 9.5, as this is the ideal pH value for coupling aldehyde groups. Monitor pH using narrow-range pH test strips.
7. Mix the solution gently for 3 h at room temperature.
8. Add 10% (v/v) 2 M ethanolamine, pH 9.6 (final concentration of 0.2 M) to the solution to block any remaining sites on the activated protein.
9. Incubate the solution overnight at 4 °C. It is not necessary to constantly mix at this stage.

3.6.4 Enzyme–conjugate purification (day 3)

1. Add an equal volume of stock-saturated $(NH_4)_2SO_4$ solution to the coupled solution and mix gently (16 rpm) for 15 min at room temperature. Centrifuge in a bench centrifuge at room temperature for 15 min (150 × g), discard the supernatant and resuspend the pellet in the original coupled volume of PBS. The pellet can be soft so take care pipetting or tipping the supernatant off. Add thimerosal (merthiolate) to a final concentration of 0.01%.
2. Dispense the conjugate solution into 1 mL aliquots in sterile polypropylene tubes and store at 4 °C.
3. Do not filter sterilise as 50% of the activity will be lost.

3.6.5 Sandwich immunoassay

1. Using a large pipette, add three drops of test sample, negative control or positive control into the wells of a microtitre plate. For every 10 samples being tested, at least one positive control and one negative control should be included. All samples should be tested in duplicate, but preferably in triplicate, including all the controls. Immediately swirl gently for 2–3 s, then allow to stand for 30 min at 37 °C.

2. Empty the wells and briefly wash three times with washing solution. Shake out remaining washing buffer and tap dry on absorbent paper.
3. Prepare an enzyme–antibody conjugate solution at 1:1,000 in PBS.
4. Add three drops of enzyme–antibody conjugate solution to each well and immediately swirl gently for 2–3 s, then stand for 15 min at 37 °C.
5. Empty the wells and briefly wash three times with washing solution. Shake out remaining washing buffer and tap dry on absorbent paper.
6. Add three drops of substrate to each well. Leave for 5 min at room temperature to allow the blue colour to develop.
7. Stop reaction by adding one drop of stop solution. Gently mix the contents of the microwells, which should now turn yellow.
8. Compare the sample test wells to the positive control wells against a white background. If the colour in the sample is a darker yellow than the colour in the positive wells, the sample contains more than that of the positive control (i.e. more than 5×10^6 cells per mL, according to our original example in Section 3.6.1). Of course, like all rapid tests, this will be somewhat subjective and therefore only semi-quantitative. However, if a plate- or strip-reader is available, each well can be read at 450 nm to give a more quantitative result.

3.7 Real-time polymerase chain reaction

Real-time PCR (qPCR) is based on comparing amplification of sample DNA to a standard DNA dilution series and back-calculating to an initial sample concentration. To be able to detect target sequences during amplification, a fluorescent dye such as SYBR Green is used, which will bind to double-stranded DNA. Alternatively, modified oligonucleotide probes can be used that will free DNA-bound TaqMan probes when complementary DNA hybridises and is then synthesised. In both cases, the signal will be proportional to the amount of DNA synthesised (Figure 3.6). The following protocol describes the SYBR Green technology.

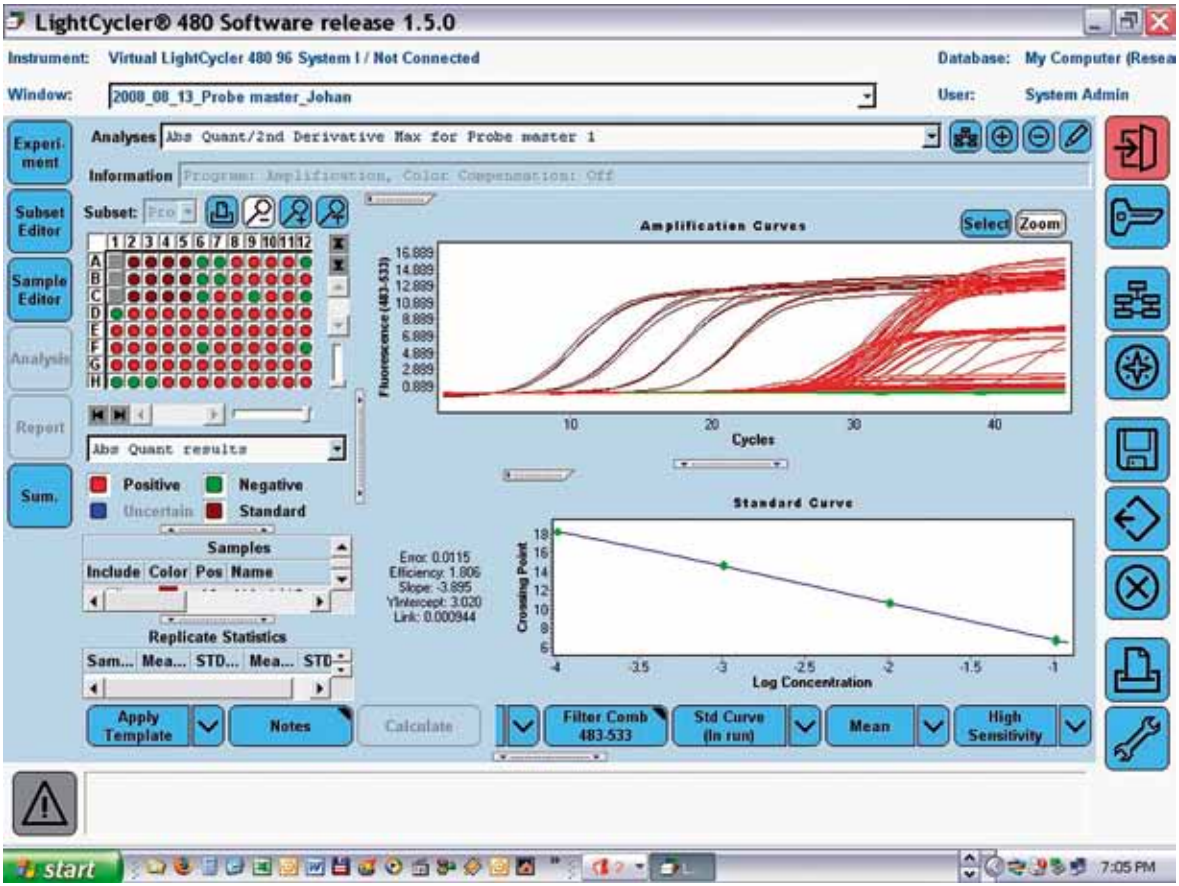


Figure 3.6 *LightCycler software screen showing detection of amplification of different concentrations of fluorescently labelled double stranded DNA. A calibration curve can be constructed from its measurement and used to determine DNA quantity in a sample with unknown DNA concentration.*

3.7.1 Reagents, equipment and services

- Commercial DNA extraction kit or simple preparation (e.g. Qiagen DNeasy kit or MoBio's PowerSoil DNA Isolation Kit)
- Oligonucleotide synthetising services (for primers)
- Primer design software (free online or purchased commercial tools)
- qPCR mastermix containing bases, buffers and DNA polymerases
- Microcentrifuge
- Gel electrophoresis apparatus
- Illuminator for visualisation of DNA bands (UV or a Dark Reader)
- DNA sequencing services
- UV spectrophotometer

3.7.2 Sample preparation

1. Grow *Azospirillum brasilense* Sp 245 or another biofertiliser bacterial strain in modified nutrient broth (MNB) or other suitable media at 28 °C with shaking (120 rpm) for 36 h.
2. Extract DNA using the chosen kit, following the manufacturer's protocol.

3.7.3 Design of primers

Based on known sequences, primers can be designed using free online tools or commercial software, or use those previously published in the literature.

In the case of *A. brasilense* Sp 245, primers were based on 23S rRNA gene sequences as recommended by Kirchhof et al. (1997):

- forward sequence 5′ CTCTGGTGCACCGGTTGT 3′
- reverse sequence 5′ GCGGTTATCCCGTCCATAC 3′.

Allow up to one week to receive the oligonucleotides from the supplier.

3.7.4 Optimisation of real-time PCR assay

A robust qPCR assay can only be achieved by optimisation of its parameters.

1. Prepare the PCR mixture using appropriate amounts of water, primers, template DNA and SYBR Green mastermix (e.g. Roche).
2. To determine optimum primer concentration, test at least two primer concentrations of 0.05 and 0.1 µM.
3. The melting curve was established by exposing the PCR products for 10 s at 95 °C, 30 s at 55 °C and back to 95 °C (in continuous mode). The qPCR amplification was carried out using a LightCycler 480 (Roche). Use the following program for amplifying *A. brasilense* Sp 245:
   - 10 min initial denaturing at 95 °C
   - 45 cycles of 10 s at 95 °C, 10 s at 60 °C and 30 s at 72 °C
   - Choose the primer concentration that gives the highest efficiency and earliest crossing point for further work.
4. For optimum annealing temperature, select a range of temperatures around the pre-determined melting temperatures of the primers. For *A. brasilense* Sp 245, 55 °C, 58 °C and 60 °C, were tested.
5. Determine the optimum concentration of template DNA from the amplification curve. Choose the value below the lowest level that still causes PCR inhibition.

6. Determine the concentration of template DNA for a standard curve using a NanoDrop spectrophotometer, using 10-fold serial dilutions.

Optimum parameters for A. brasilense *Sp 245*

The DNA concentration was 5.28 ng/µl. The qPCR amplification cycle for constructing this standard curve was:

- 10 min initial denaturing at 95 °C, followed by 45 cycles of
  - 10 s at 95 °C
  - 10 s at 60 °C
  - 30 s at 72 °C.

The melting curve was established by exposing the PCR products for 10 s at 95 °C, 30 s at 55 °C and back to 95 °C (in continuous mode). The primer concentration used in this experiment was 0.1 µM. A good standard curve should have an efficiency value close to 2, which indicates a doubling of the original sample (template) DNA in each cycle.

3.7.5 qPCR-assisted count of soil bacteria

1. Extract the total DNA from soil samples using PowerSoil DNA Isolation Kit (MoBio), following the manufacturer's directions.
2. Store the DNA samples at –20 °C.
3. Thaw the DNA samples and perform qPCR on them as described above. Determine the number of target micro-organisms per 1 g of soil as follows:
   a. The standard curve created in Section 3.7.4 plots the logarithm of the original DNA concentration (ng/µL) against the 'crossing point', which is determined by the RT-PCR software for every sample. The original DNA concentration should be converted to 'gene copies' per reaction mix (GC per RM) according to the formula

      GC per RM = DNA concentration (ng/µL) × 10^{-9} (ng/g) × 1/660 (mol bp DNA/g DNA) × (6.023×10^{23}) (bp/mol bp DNA) × 1/(genome or plasmid size, bp) × volume of template (µL)

   b. Determine the gene copies per g of soil using the formula

      GC per g soil = [GC per RM × volume of DNA (µL)]/ [3 (µL DNA per RM) × soil mass (g)]

Combining tests for viability with immunological methods

We have attempted to develop methods that are specific, quantitative and indicative of viability. These have usually involved a modification of immunological methods so that viability and sensitivity can be improved. In some cases, the testing time has been reduced considerably; however, questions remain about specificity.

Lupwayi et al. (2000) reviewed several immunological methods for counting viable rhizobia in legume inoculants. The review included agglutination tests, immunoblotting techniques (spot blot and colony-lift immunoblotting), indirect fluorescent antibody microscopy and a syringe-filter enzyme immunoassay. The spot blot is useful for detecting the presence of the correct strain and the other techniques are useful for enumeration. The syringe filter method uses a colorimetric assay to determine cell density. Methods have also been developed using immunomagnetic beads to retrieve specific cells from mixed cell samples (Dye 1994; Olsen and Rice 1996). Dye (1994) first reacted rhizobial cells with antibodies raised in rabbits and collected cell-antibody conjugates using immunomagnetic beads coated with sheep anti-rabbit antibodies. Cell–antibody–bead complexes were then diluted in sterile deionised water and spread onto the surface of yeast mannitol agar medium. He recovered 55% of the cells after a 45–60 min incubation with the immunomagnetic beads.

By using differential nucleic acid stains, Olsen and Rice (1996) were able to reduce the amount of time required to count bacteria in inoculants. The cells were collected from peat using biotin-conjugated monoclonal antibodies and streptavidin-labelled magnetic beads. Using this method, cell recovery ranged from 23.9% to 51.1%, and the whole procedure took only 90 min. Compared to MPN assays from a plant infection test, this is an enormous time reduction.

At the University of Sydney, Australia, researchers evaluated the efficiency of cell collection by immunomagnetic bead separation. At high *Citrobacter freundii*–bead ratios, complexes formed and resulted in underestimated cell numbers when confirmed by plate count. The method was modified to include a multiple-tube component where the presence or absence of cells was determined after antibody or antibody–magnetic-bead capture. Viability of captured cells was then determined using a differential carbohydrate method. The results were variable, and non-specific binding was a major problem. Further work could investigate the recovery after antibody – cell agglutination before immunomagnetic separation. Flow cytometry may also be used to enumerate live and dead cells after differential nucleic acid staining.

04

Quality control of biofertilisers

To maintain a high-quality product there should be an effective quality assurance or quality control program. Quality control can be defined as the process of measuring defined quality parameters of a product. Quality assurance is an overall check that quality control procedures and techniques are achieving what they intend to achieve.

To achieve adequate quality control of a product, quality needs to be defined by establishing standards. Standards may incorporate a number of parameters relating to what is known about product efficacy. Most of our understanding of microbial inoculant quality comes from extensive studies of legume inoculants and their effective application to crops. Through this work, numerical standards were developed as key indicators of product quality from observations that product efficacy was proportional to the number of viable cells delivered by inoculation. Although it is assumed that the number of viable cells is important for efficacy of biofertilsers, numerical standards for non-rhizobial inoculants are generally not well established. Standards presented here for BioGro are based on field observations; however, it is expected that these may vary depending on the microbial species and strains applied.

There are several points during the production of biofertilisers where tests can be done to determine quality. Tests on the original cultures or 'mother' cultures should ensure purity and function of selected micro-organisms before producing starter culture for distribution. Any problems at this point can be easily rectified. Checks throughout production on starter cultures and final products are also essential so that any problems in production can be quickly identified.

4.1 Maintenance and distribution of biofertiliser strains

Supply of mother cultures from a single source is preferred so that variation is minimised. Ideally, mother cultures should be preserved to minimise genetic drift. Cultures may be maintained on culture media and refrigerated; however, regular subculturing may allow genetic drift and ultimate loss of plant growth-promoting function. Strain stability is improved by freezing after suspending in cryoprotectants such as glycerol (usually at about 15–20% v/v) or freeze-drying after suspension in protective media that includes nutrients like peptone (5%) and sucrose (10%). When preserving cultures, genetic drift can be minimised by using a parent/working-lot system (Figure 4.1). This system allows early cultures to be available for production of new working stock. In this example, parent culture lots are prepared from an initial culture and used to prepare several working cultures. The first set of working cultures can be made from the original culture. In the context of inoculant production, parent lots should be prepared and stored as mother cultures, and working lots prepared and distributed for starter culture production used in factories to prepare commercial products. Ideally, culture collections should be replicated and stored at different locations to reduce the risk of loss.

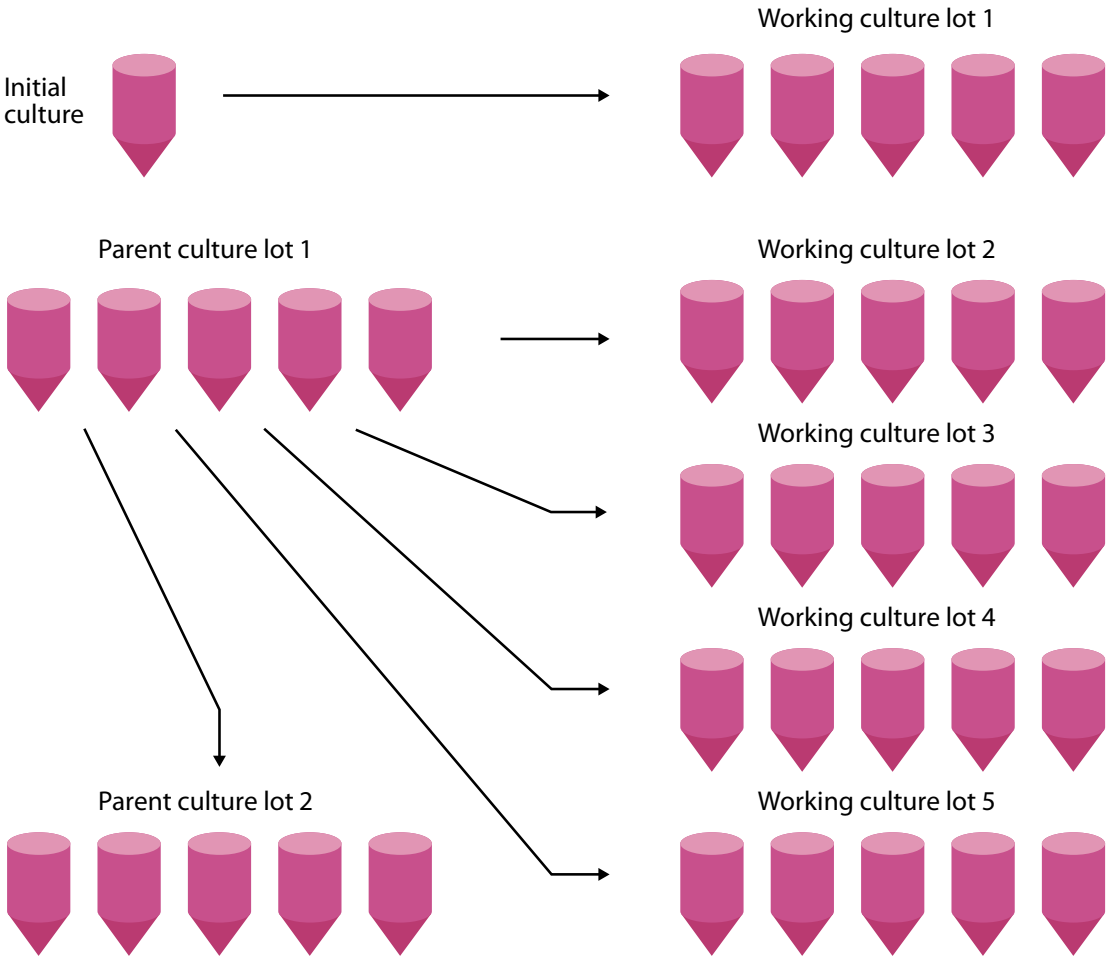


Figure 4.1 *Parent/working-lot system for culture preservation. Adapted from 'Thermo Scientific Nalgene and Nunc cryopreservation guide', available at <www.thermoscientific.com>.*

Microbial strains used in the production of BioGro

Before 2005, BioGro contained three strains of bacteria (1N, 4P and 3C) selected from rice rhizospheres in the Hanoi area of Vietnam (Nguyen et al. 2003). Since 2005, a new combination has been used from four strains—1N, HY, B9 and E19—based on more reliable field results. Strain 1N (*Pseudomonas fluorescens*) was originally selected for its ability to grow on nitrogen-free medium as an indication of potential nitrogen fixation. However, it has recently been shown to produce indole acetic acid (IAA), the enzyme ACC deaminase, as well as siderophores. The soil yeast HY (*Candida tropicalis*) was selected for its ability to solubilise insoluble PO_4 in agar medium. Two other bacterial strains, B9 (*Bacillus subtilise*) and E19 (*Bacillus amyloliquefaciens*), were selected for their ability to degrade protein, cellulose and starch. All four strains currently used in BioGro have been observed to exert some plant-growing production effects, and it is also possible that the bacteria exert biocontrol effects when pathogens are present. Maintenance of function can be tested using methods described in Chapter 1.

Storage media used in Vietnam for maintenance of BioGro cultures (prepared as agar slopes for storage, which may be covered with sterilised paraffin oil for longer shelf life) are listed below.

Strain	**Recipe for 1 L media (adjusted to pH 7.0)**
HY (Pikovskaya 1948)	10 g glucose; 5.0 g $Ca_3(PO_4)_2$; 0.2 g KCl; 0.2 g NaCl; 0.5 g $(NH_4)_2SO_4$; 0.002 g $MnSO_4$; 0.002 g $FeSO_4$; 0.1 g $MgSO_4.7H_2O$; 0.5 g yeast extract; 20 g agar
1N	30 g/L tryptic soy broth; 15 g/L agar
B9 and E19	10 g/L peptone; 3 g/L tryptone; 3 g/L NaCl; 15 g/L agar

4.2 Carrier materials for inoculant production

Peat is the most common form of carrier for microbial inoculants; however, many alternatives have been investigated (Figure 4.2). A description of the main steps for the production of BioGro is presented in the box on the next page. For every new source of peat or comparable carrier material, tests must be undertaken to determine the suitability of the carrier for the particular biofertiliser strains. Peat or high organic-matter soil should be drained after being excavated and allowed to air dry before testing for compatibility with micro-organisms. Several carrier properties affect microbial growth and survival, and—ultimately—the quality of inoculant products. Major factors are listed below, but there may be many other factors affecting the quality of peat carrier. The ultimate measure is the growth and survival of inoculant micro-organisms.

4.2.1 Adjusting the pH of peat

First, determine the pH of the carrier and if necessary adjust it to pH 7.0 with $CaCO_3$ before inoculation (unless the strain to be used has a special pH requirement). If $CaCO_3$ is required, it should be well mixed and reacted with the carrier carefully, allowing time for equilibration. The reaction between $CaCO_3$ and H^+ in peat will depend on moisture and particle size. The amount of $CaCO_3$ required to change the pH will depend on organic matter and clay content, as well as the buffering capacity of the peat. Finely milled agricultural lime (calcium carbonate with some impurities passed through a 150 µm mesh) is the best limestone to use to adjust pH. Builders lime is highly caustic and should be used with caution and other forms of lime may be too weak.

Figure 4.2 *Different biofertiliser carrier materials*

Manufacture of peat starter culture for BioGro

It is essential that peat starter cultures, supplied for use in factories to prepare commercial biofertilisers, have sufficiently high numbers of viable cells of each individual species and be as pure as possible. The different species are kept separate during solid-state fermentation stages and mixed at the end just before the packaging process. Where non-sterile carrier media are used (e.g. peat or composted organic materials like filter cake from sugarcane or sludge from aquaculture), starter cultures with high numbers of fast-growing microbes will be more likely to outgrow resident microbial populations in the carrier.

For preparation of starter culture, each of the four bacteria is grown in a separate broth culture. They are then individually added to the carrier formulated by mixing peat (about 75% by weight, depending on moisture content) with broth culture, plus water and a source of sugar (1% w/w). The well-mixed culture is incubated for 48 h at about 30 °C. The starter cultures are then multiplied 10-fold in local factories by mixing with fresh peat and sugar as before, aiming for optimum moisture content for growth in the final product. For final packaging of the BioGro product, peat cultures of all four strains are mixed in equal ratios by weight.

Quality control should ideally be carried out on the initial mother cultures, starter culture and final product immediately after preparation and during storage to check the stability.

Masks should be worn when mixing carrier material with starter culture

Media for fermentation should be readily available, consistent in quality and affordable, given large quantities are required for commercial-scale fermentations. Fermentation media for BioGro production in Vietnam are listed below.

Fermentation media for biofertiliser strains

Strain	Recipe for 1 L media (adjusted to pH 7.0)
HY	Same as the storage media, but without agar
1N	5.0 g/L peptone; 15 mL fish sauce
B9 & E19	10 g/L peptone; 20 mL fish sauce

4.2.2 Population density of indigenous microbial population

Before inoculation, the initial number of indigenous bacteria in the peat should be determined using general heterotrophic microbiological media (glucose peptone media is useful for this). The number should be at least 100-fold lower than the number introduced in the inoculum (calculated per g of carrier). Generally, inoculant carriers for biofertiliser production are not sterilised before production because of the large quantities required. Therefore, high levels of indigenous micro-organisms may reduce optimal growth of the selected biofertiliser strains. This may occur directly through competition for resources or indirectly through production of antagonistic metabolites such as antibiotics.

4.2.3 Moisture potential

The moisture potential of inoculant will vary according to carrier properties and is important as it affects microbial growth and survival. Particle size, organic matter and clay content of peat play a role in water-holding capacity and water potential. It is desirable to increase the water-holding capacity of the peat so that larger amounts of broth culture (and hence more cells) can be introduced to peat before incubation and so that moisture potential is optimum for growth. Moisture potential can be difficult to calculate, and growth of micro-organisms should be determined at a range of moisture contents to find the optimum level. Examples of experimental designs to determine optimum moisture content are described in Section 4.3.

4.3 Experimental designs to define quality parameters of peat-based inoculant

4.3.1 Optimum moisture content for growth and survival of inoculant micro-organisms

Before injecting peat with broth it should be sterilised (e.g. by autoclaving or gamma sterilisation) after adjusting the moisture content to 20%. This will minimise variation from confounding affects of indigenous micro-organisms. The efficacy of sterilisation should be measured by inoculating sterilised peat with sterile deionised water or nutrient broth and measuring the growth of any contaminants for one month. To do this, suspend peat in sterile deionised water, dilute and spread onto the surface of glucose–peptone media. Record the dilutions at which growth occurs.

To determine the optimum moisture content for the growth and survival of biofertiliser strains, experiments should be designed that vary the moisture content without changing the inoculum size of the microbial strain added to peat. This is done by varying the volume of sterile water or broth added to peat while maintaining a constant volume of culture broth. Examples of experimental treatments are listed in Table 4.1.

Table 4.1 *Examples of treatments for measuring optimum moisture content for microbial inoculants*

Moisture content (%)	Liquid added (mL)	Volume of broth (mL)	Volume of sterile deionised water (mL)
40	29.5	29.5	0
50	52.5	29.5	23
60	87.5	29.5	58

Note: Calculations are based on 70 g dry peat after adjusting to 20% moisture content for sterilisation.

The number of viable cells per g of carrier should be measured at several intervals to indicate the relative growth and survival. Biofertiliser strains should be clearly differentiated from other organisms using techniques described in Chapter 3 and later in this chapter.

4.3.2 Calculating the amount of liquid to add to peat or carrier mixture

The equation below is used to calculate moisture content of 70 g dry peat

$$\frac{x}{70 + x} = \frac{y}{100}$$

where x is the amount of liquid added and y is the final moisture percentage (e.g. 50%).

The same equation can be used for any quantity of peat, but as peat will invariably already contain some moisture, the mass of dry peat must first be calculated.

To adjust 70 g of dry peat to 20% for sterilisation, 17.5 mL of deionised water should be added. An example calculation is shown in the adjacent box.

Example calculation of liquid added to peat to obtain specific moisture content

How much liquid (e.g. broth) is added to 150 g peat with 20% moisture to get a final moisture content of 35%?

Mass of dry peat:

$$\frac{20}{100} \times 150 \text{ g} = 30 \text{ g}$$

$$150 \text{ g} - 30 \text{ g} = 120 \text{ g}$$

Moisture to add to dry peat:

$$\frac{x}{120 \text{ g} + x} = \frac{35}{100}$$

$$x = 0.35(120 \text{ g} + x)$$

$$x = 42 \text{ g} + 0.35x$$

$$x - 0.35x = 42 \text{ g}$$

$$0.65x = 42 \text{ g}$$

$$x = 64.6 \text{ g}$$

Therefore, 64.6 g moisture should be added to 120 g dry peat to achieve 35%. If peat already has 30 g moisture then 64.6 g – 30 g = 34.6 g should be added to 150 g peat.

4.4 Standards for biofertilisers

The potential range of biofertilisers is too large for one standard to be set. Rather, a standard for each product or product type may be required (see the box on the next page). Again, because biofertilisers interact with the environment, including the agricultural system, this also has to be considered when setting standards. In the case of biofertilisers, unlike legume inoculants, there is often no observable effect. Therefore, carefully designed trials are required to measure responses. Often, the mode of action of biofertilisers is unknown, and investigation of a nil response is difficult. Because many of the organisms are part of the normal population of soil organisms without distinct characteristics, it is often difficult to count their numbers in the plant rhizosphere. Again, this is a disadvantage when investigating the failure of a crop to respond, or when trying to set inoculation doses to ensure good rhizosphere colonisation.

Therefore, to set standards for biofertilisers we need to:

1. accumulate response data from field experiments
2. carefully characterise the trial sites, as described above
3. use identified and characterised strains
4. check that cultures are pure
5. count the cfu/g of active bacteria
6. calculate the inoculum potential.

Then, after accumulating sufficient data, perhaps obtained by different laboratories or field stations, it should be possible to judge the requirements of each biofertiliser matched to crop and environment in terms of:

- under what conditions was there a positive response?
- what inoculum potentials are associated with inoculation successes and failures?
- what other crop inputs are associated with successes and failures?

4.5 Quality control of biofertiliser products

The number of packets that should be tested per batch depends on the variability of the product. Ideally, representative sampling should be determined by measuring numbers of viable cfu in packets until the cumulative mean has stabilised. Other quality control systems (for legume inoculants prepared using sterile carrier) are designed so that seven packets are submitted from each batch. A subset of five packets is tested first and if one or two packets fail the other two packets are tested. If the two additional packets pass then the batch is passed (i.e. five packets out of seven must pass standards for a batch to be passed).

There should be an adequate recording system so that testing of each batch can be tracked and reported back to manufacturers. Examples of reports are presented in Appendixes 1 and 2. A preliminary report may be used to record presumptive tests and a follow-up report may record confirmatory tests. If product release is based on presumptive tests only, the logistics of product recall should be considered.

Standards for BioGro

In Vietnam, standards already exist for biofertilisers, but these are based on functional tests (e.g. phosphate solubilisation or nitrogen fixation) and are not specific to selected strains. For BioGro, standards for each particular species have been determined based on both agronomic performance and numbers per g achievable at commercial-scale production. These are expressed as colony forming units (cfu) per g of product. The current standards for each species in BioGro are listed below.

BioGro strain	cfu/g of carrier	kg/ha	Nominal cfu/ha
1N	10^7	100	10^{12}
HY	5×10^6	100	5×10^{11}
B9	10^7	100	10^{12}
E19	10^7	100	10^{12}

Numerical requirements and product efficacy depends on environmental conditions and soil properties. Application rates will vary with different methods of application, such as banding in seed rows or broadcasting by hand. Optimum inoculation rates have not been firmly established; however, most field trials to date have indicated that benefits are not increased at rates above 100 kg/ha when cell numbers are between 5×10^{11} and 1×10^{12}/ha.

Although carrier is essential for adequate cell distribution, quantity of carrier must be balanced with cost of applying large amounts of biofertiliser. To highlight the difficulties with cell distribution, it is worth noting that if liquid medium was completely removed from 10^{12} cells, the volume occupied by the cells would be little more than 1–2 mL. Clearly, this would be impossible to distribute over one hectare without the use of carrier. It is important that standards are regularly reviewed as more information becomes available.

Once the products arrive in the laboratory, they should be stored in appropriate conditions (e.g. in the refrigerator) and quality control tests should be promptly carried out. To facilitate this, it is useful if the lab staff knows the number of samples being sent ahead of time, so they can prepare the correct amounts of reagents and other materials.

The number of organisms in peat-based inoculants may be determined by preparing 10-fold dilutions in sterile deionised water (or some other suitable diluent), and plating on selective or differential media. The identity of colonies presumed to be inoculant strains can then be confirmed using specific tests (see Figures 4.3 and 4.4 for general flow diagrams, and Tables 4.2 and 4.3 for tests and media).

To complete the initial 10-fold dilution, it is necessary to determine the relative quantities of peat and diluent. To do this, the volume of peat inoculant (e.g. 10 g) at the appropriate moisture content should be determined by measuring the displacement of water in a measuring cylinder. This volume may vary for different carriers. For example, 10 g of peat may only occupy 5 mL, in which case 10 g of peat should be added to 95 mL water so that the total volume is 100 mL. This will be the 10^{-1} dilution. The number of dilutions required after that will depend on the number of micro-organisms per g of peat.

For example, if there is 10^7 cfu/g peat, the 10^{-4} and 10^{-5} dilutions should be sufficient to reduce colony numbers to 100 and 10, respectively, when 100 mL is used to inoculate plates. If the quality-control laboratory is only interested in whether a product meets standards, plating at these dilutions only would be adequate. However, during product development or when it is otherwise important to determine numbers present in the inoculant, plating of lower dilutions is necessary.

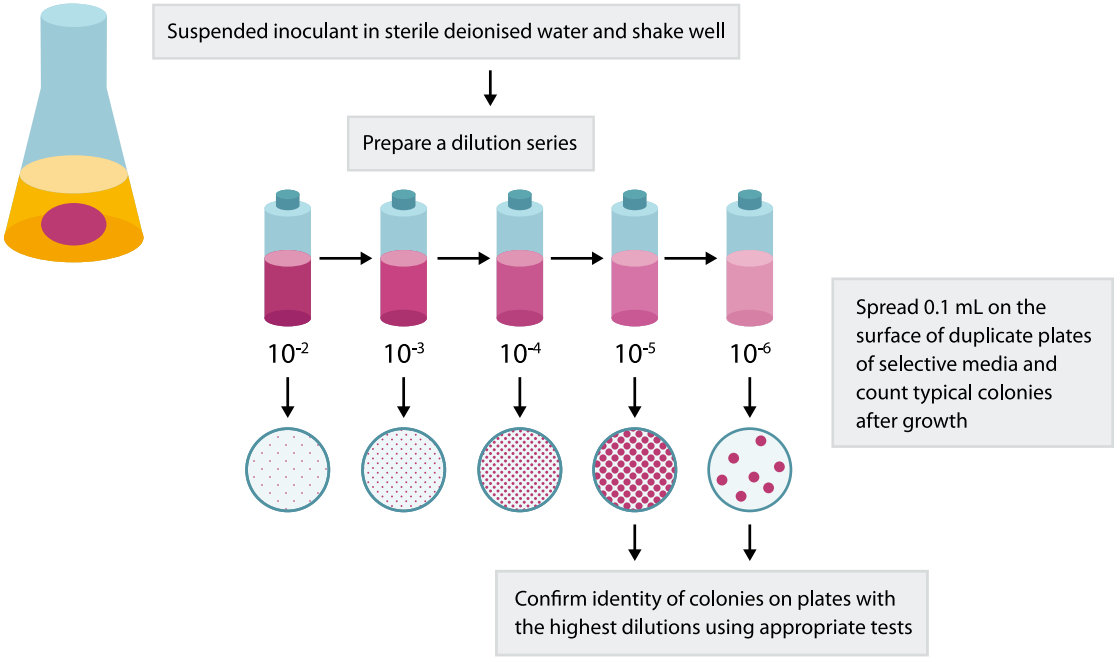


Figure 4.3 *Schematic diagram of counting numbers of viable cfu from peat inoculants. A random selection of colonies presumed to be selected inoculant strains should be confirmed using appropriate tests.*

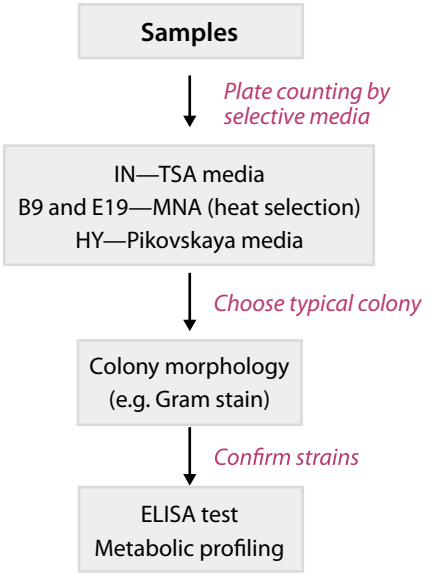


Figure 4.4 *Flow diagram for quality control of BioGro indicating initial counting using selective media for presumptive tests, and morphological, immunological and metabolic characterisation for secondary and confirmatory tests*

Table 4.2 *Suggestions for selective media and confirmatory tests for BioGro strains*

Strain	Selective/ differential media	Confirmatory tests
1N	Tryptic soy or nutrient agar with antibiotics (vancomycin and cycloheximide)	Gram stain Antibody reaction using colony immunoblotting or ELISA
HY	Pikovskaya medium for P-solubilising colonies (look for zone of clearing around colony) or selection using nutrient agar with tetracycline	Gram stain Antibody reaction using colony immunoblotting or ELISA Sensitivity to cycloheximide
B9	Nutrient or modified nutrient agar and heat selection at 80 °C, or colony morphology	Gram and spore stain Ability to suppress growth of *Fusarium* sp. but not *Bradyrhizobium japonicum* CB1809
E19	Nutrient or modified nutrient agar and colony morphology	Gram and spore stain Ability to suppress growth of *B. japonicum* CB1809

ELISA = enzyme-linked immunosorbent assay; P = phosphorus

Table 4.3 *Media recipes for plate-counting BioGro strains*

Strain	Recipe for 1L media
HY	Pikovskaya recipe listed in the box on page 64. Nutrient agar (3 g beef extract; 5 g peptone; 15 g agar) Antibiotics (tetracycline, 10 mg/L)
1N	Nutrient agar Antibiotics (vancomycin, 10 mg/L and cycloheximide, 100 mg/L)
B9 and E19	Nutrient agar supplemented with 5 g yeast extract, 0.5 g glucose, 0.5 g sucrose

4.6 Occupational health and safety for Biofertilisers

The ethical manufacture of good-quality biofertiliser products requires both safe production and safe use in agriculture (see the box on the next page). This demands that the manufacturing procedures and selected micro-organisms must have passed a rigorous risk assessment, considering the following factors:

- All biofertiliser micro-organisms used should be known to be non-pathogenic to humans, animals or plants. Obviously, this requirement demands that they be identified by genus and species as described elsewhere in this manual. In normal practice, this will mean that if there is significant doubt about the identification of any microbial strain, this strain should not be used, irrespective of how successfully it promotes plant growth and crop yield.
- All micro-organisms to be included in biofertiliser products should be shown to be compatible with each other, even if they are grown separately in production.
- Information on whether there are incompatibilities between biofertiliser micro-organisms and other beneficial strains of microbes that farmers may need to use (such as rhizobia for inoculating legumes) should also be prominently displayed, preferably on the label.
- Inert carrier materials should also be shown to be of good quality, free from organic contaminants, faecal coliforms, heavy metals or other undesirable properties (e.g. acidity—adjust with limestone). Such materials should be handled to minimise dust generation with effective face masks worn by workers.
- Safeguarding human health should be a strict operating principle in biofertiliser factories, including protection from heavy lifting and other hazards.

Biosafety for BioGro

All the principles described in Section 4.6 have been applied in the selection of suitable plant growth-promoting strains and the factory production of BioGro.

For example, before exact strain identification, earlier versions of BioGro, which established the effectiveness of inoculant fertilisers in promoting rice yields, included two species later considered to be minor hazards. These were identified as *Klebsiella pneumoniae* (4P) and *Citrobacter freundii* (3C). Although no effects on human health were ever observed and the likelihood that these particular strains would act ever as pathogens—except with hospitalised immuno-deficient individuals—is slight, they are no longer included in BioGro. However, they are considered as safe under laboratory conditions and continue to be studied by researchers for their plant growth-promoting properties.

In another case, *Burkholderia vietnamensis* was shown to be a promising plant growth-promoting strain, as it had similar benefits to BioGro for the growth of rice in extensive field experiments in Vietnam (Gillis et al. 1995; Van et al. 2000). When this strain was proposed to be included in a plant growth-promoting biofertiliser product in North America, it was ineligible for registration because of its close relationship to *Burkholderia cepacia.* This micro-organism has been shown to accentuate cystic fibrosis, although only in very rare cases with immuno-compromised patients.

05

Quality control of biofertiliser action in the field

For their acceptance as new technology, biofertilisers must easily demonstrate their reliability in the field. Effectiveness in laboratory or greenhouse conditions is insufficient; farmers need to see improved productivity of their crops before they can be expected to adopt and continue to use these products. Instructions for application should be clearly displayed on product packaging.

Some of the important questions farmers or extension agents are likely to ask include:

- What is the effect of the rate of application, and how little or how much product should I add per unit area? The rate of application required for a positive plant-growth response will be dependent on physical, chemical and biological conditions of soil at each site. Recommended application rates should deliver numbers of organisms most likely to give a response in a variety of conditions.
- What rate of other fertilisers (nitrogen and phosphorus) should be used and how much can this be reduced with added biofertiliser? The general principle is that use of biofertiliser should enable a reduction in the rates of fertiliser application. Importantly, biofertiliser may have reduced effectiveness if high rates of chemical fertiliser are maintained, because of possible repression of beneficial organisms.
- How often should the biofertiliser be added? There may be grounds for a second application if the rice crop has not responded to the first application, but more measurements are needed to answer this question.
- What is the effect of agrochemicals, such as herbicides and fungicides, on the effectiveness of the biofertiliser? Information on possible interference needs to be provided with the product or on the label.

Package labelling instructions for use of BioGro

The answers to these questions will vary depending on the organisms contained in the biofertiliser, along with the specific soil and environmental conditions at the site of application. So, to improve the performance of biofertilisers, it will be useful to record the results of variations in the mode of application. It will also be important to adopt certain protocols to confirm the plant growth-promoting effect of the product in the field, and to verify its effect on yield through strip trials. There is no satisfactory substitute for a field test when assessing strains or when determining the best way to integrate them into an agricultural system. Field tests are usually expensive and so must be carefully planned. They may differ in terms of complexity and the demand made on resources. In order of complexity, we have used:

1. simple plus–minus experiments managed cooperatively with several farmers
2. simple replicated comparisons of strains arranged in a randomised block design
3. more complicated split-plot designs. These are used to study the interaction of a chosen strain or a multistrain biofertiliser with, for example, rates of other nutritional inputs (e.g. nitrogen or phosphorus) with different splits, or use of farmyard manure.

Examples of the simplest through to the most complicated trials are detailed below.

5.1 Nursery testing of plant growth promotion

Despite quality control throughout the production and supply chain of microbial inoculants, the possibility remains that a certain product will not perform to expectation. The best and most important test of a biofertiliser product will always be in the field under local environmental conditions. Farmers should always endeavour to test the product first on a small scale, to be confident that it will work as guaranteed and ensure good economic return.

The biofertiliser should be applied with rice seed planted in a soil nursery at the rate of 40 kg biofertiliser per ha (4 g/m^2), or as otherwise recommended. A control nursery with no added biofertiliser should also be established.

After 3 weeks growth, seedlings should be removed gently from the soil and washed with water. A visual comparison should reveal significant differences between the control and the test plants. Differences in roots or shoots may be verified by fresh or dry weight measurements.

Demonstration of the plant growth-promoting effect of BioGro on rice at the seedling stage; BioGro-treated seedlings on the left (left-hand photo) and BioGro-treated seedlings on the right (right-hand photo). The root systems of BioGro-treated seedlings were noticeably more vigorous than those of untreated seedlings.

5.2 Strip trials on rice farms

Strip trials have been particularly useful, as they have allowed the testing of a biofertiliser across a range of sites and under normal farming conditions. These are not replicated experiments in the normal sense, but with farmers' cooperation, a number of comparisons can be made that lend confidence to the results. The trials consist of dividing a farmer's land into two halves. On one half, the nutrient inputs are halved and biofertiliser added. On the other, control half, normal nutrient inputs are added, but with no biofertiliser. Each area is harvested separately. Before the trials are established, the participating farmers should be asked to complete a form to provide background information (e.g. Table 5.1).

An example of the information that might be expected from such a 'plus–minus' trial is shown in Table 5.2. This group of farmers obtained a consistent response to biofertilisers with a mean yield increase of 15%, which represented a total benefit of 46,000 VND. Such a trial provides not only information about the efficacy of the strain, the conditions under which it is effective as well as information of other input requirements, but is also a major extension tool.

Table 5.1 *Questionnaire for farmers cooperating for strip-plot trials*

Pre-planting data		**Details**	**Pre-planting data**		**Details**
Site details	Village		**Biofertiliser details**	Strain combination	
	Farm/ farmer name			Carrier type	
	Soil type/texture			Date of manufacture	
	Soil pH			Quality control (yes/no, cell no.)	
	Previous crop			Application method	
	Previous fertiliser			Date of application	
	Previous biofertiliser			Amount applied	
Strip-trial data		**Details**	**Strip-trial data**		**Details**
Plot with biofertiliser	Plot area		Plot without biofertiliser	Plot area	
	Nitrogen (type, amount)			Nitrogen (type, amount)	
	Phosphorus (type, amount)			Phosphorus (type, amount)	
	Potassium (type, amount)			Potassium (type, amount)	
	Farmyard manure (type, amount)			Farmyard manure (type, amount)	

Table 5.2 *Effect of biofertiliser on the yield of rice and farm profits for 10 farmers near Hanoi*

Farm	Yield (kg/sao)[a]		Yield increase (kg/sao)	Value (VND)	Cost of fertiliser inputs (VND/sao)		Saving (VND/sao)	Total economic benefit (VND/sao)
	– BF	+ BF			– BF	+ BF		
1	194.4	234.0	39.6	59,400	73,400	66,700	6,700	66,100
2	183.6	234.0	50.4	75,600	73,400	69,500	3,900	79,500
3	194.4	223.2	28.8	43,200	73,400	71,900	1,500	43,700
4	208.8	223.2	14.4	21,600	70,400	66,400	4,000	25,600
5	180.0	223.2	43.2	64,800	73,800	72,800	1,000	65,800
6	172.8	201.6	28.8	43,200	71,500	72,900	–1,400	41,800
7	180.0	201.6	21.6	32,400	85,200	76,900	8,300	40,700
8	172.8	187.2	14.4	21,600	61,500	60,900	600	22,200
9	165.6	187.2	21.6	32,400	76,300	71,000	5,300	37,700
10	180.0	201.6	21.6	32,400	83,400	70,700	12,700	45,100
Mean	**183.2**	**211.7**	**28.4**	**42,660**	**74,230**	**69,970**	**4,260**	**46,820**

BF = biofertiliser; VND = Vietnamese dong
a 1 sao is equivalent to 360 m^2

5.3 Field experiments to investigate the interaction between biofertiliser and other inputs

More complex field experiments are necessary to really optimise the agronomic application of biofertilisers, and to tease out which environmental, agronomic and economic factors have the biggest influence on successful or unsuccessful application. In these circumstances, a good knowledge of multivariate statistics is recommended in order to identify multiple correlations and covariation among predictor and response variables. The following text gives some practical advice about conducting these types of larger field trials, and Appendixes 3 and 4 suggest some further considerations to the design of the experiment and parameters needed to evaluate such trials.

5.3.1 Selecting a suitable site

It is important to select a site where the conditions are as uniform as possible. If there is some variation, place each replicate on a uniform area. The soil type should be identified, the soil sampled and the pH determined using a dilution of 1 part soil to 2.5 parts 10 mM $CaCl_2$. Where possible, the total N and NO_3 concentrations should be determined. The previous cropping and fertiliser history must be known and recorded. The site should not be prone to flooding in a normal year. If flooding does occur, good drainage should be in place to remove the water as soon as possible. It is desirable to have good access for ease in taking materials and removing samples.

5.3.2 Preparing the site

When preparing sites for rice experiments involving micro-organisms, it is essential that problems of cross-contamination between treatments are anticipated and the site prepared accordingly. This requires care in designing the plot layout but also requires the construction of banks between treatments, which will prevent water flow from one plot to another.

Experimental site showing excellent banks in Dr Cong's Trial at Cu Chi, Vietnam, in 2004

5.3.3 Applying the treatments

The most important aspect of investigating biological materials in field trials is to contain the material to its own plot. This is particularly important with biofertilisers, because if there is spread by the bacteria to other plots the results will be confounded. Rice fields' requirement for the plots to be flooded is an added complication. Water is a good agent for spreading microbes from plots inoculated with biofertiliser to uninoculated plots. Therefore, it is essential, as indicated in Section 5.3.2, to construct higher and wider banks than normal to separate the plots and prevent water from spreading to adjacent

plots. Water control is particularly important in the early stages of growth when the rhizosphere population of micro-organisms is establishing. It is also important to control drainage so that it is directed well away from the plot area.

It is important to ensure that biofertiliser is not spread between plots by farm workers, or by implements and animals. This may require separate buffalo for each biofertiliser treatment, and separate workers and implements to transfer rice seedlings and to weed the plots of each biofertiliser treatment. Where these precautions are not possible it is essential that implements and farmer's hands, feet or shoes be cleaned and sterilised with disinfectant before changing treatments.

To avoid contamination, first apply all fertilisers or other nutrients before any biofertiliser is applied. Transplant rice in uninoculated plots first. Allocate a person to be responsible for sowing a treatment. This is not normal recommended practice, but aids in reducing cross-contamination. Make sure that everyone walks only on soil barriers when moving about the plots. If it is necessary to walk on the plots, sterilise shoes immediately. It is important that all fertiliser treatments be weighed accurately and applied evenly. When amounts of fertiliser are small, they may be mixed with dry sand to increase their bulk.

Farmers transplanting rice against marked string on the side bank

5.3.4 Weeding

Similar precautions should be taken as described in Section 5.3.3, 'Applying the treatments'. It is necessary to ensure that all precautions are taken to avoid cross-contamination on shoes, implements and so on. These should all be cleaned and disinfected in between moving to another plot.

5.3.5 Sampling

Precautions to restrict contamination as described in the above two sections are also necessary at this stage and, as much as possible, supervision by extension officers or researchers is recommended (Appendixes 3 and 4 for more details). The time of sampling and the samples taken would vary according to the questions asked in the experiment. It would be usual to sample for plant height, shoot dry weight and root dry weight.

Scientist and farmer cooperate at harvest at Da Moi

Professor Hien drying the rice harvested from Da Moi

5.3.6 Harvesting

Grain should be dried before measurement and the amount of grain should be expressed at a moisture content of 12%. Extra care should be taken to ensure a uniform population of rice plants to reduce the variability of the estimates of yield. We established 45 hills of rice per m^2 by marking out each row and planting at intervals marked on a measuring stick. This enabled our grain harvest to be based on 45 hills and the results to be expressed on an area basis. This increased the uniformity of the estimates of yield, which are otherwise varied due to the difficulty in taking quadrants and the differences in plant density. We harvested 10 lots of 45 hills per plot.

5.3.7 Recording results

Recording results is very important. It will often be someone other than the experimenter who has to analyse the data. A suggested checklist is given in Appendix 3. Make sure all the units are recorded with the data (e.g. kg/sao)—never assume the person analysing the data knows the units.

5.3.8 Example field experiment

In the experiment outlined in the box below, the main questions asked were: is there a response to treatment with biofertiliser, and is there an effect of rate of application between 2 and 8 kg/sao? Also, does the rate of farmyard manure (FYM) influence any response when a basal dressing of N, P, K is applied?

Example field experiment

Design: Split plot with whole plots for levels of FYM and subplots for levels of biofertiliser.

Replication: Four replicates, two at each of two farms. Replicates 1 and 2 are adjacent, and about 9 m from replicates 3 and 4.

Treatments:

1. Four levels of biofertiliser (0, 2, 4, 8 kg/sao) equivalent to 0, 55.5, 111 and 222 kg/ha (1 sao = 360 m^2).
2. Three levels of FYM (200, 300, 400 kg/sao) equivalent to a wet weight of 5,560, 11,120 and 22,240 kg/ha.

Plot size: 16 m × 2.5 m (40.0 m^2).

Rice variety: As applicable.

Planting time: As applicable.

Planting geometry: 45 hills/m^2 with three plants per hill.

Basal fertiliser for experimental area:

- urea—3 kg/sao (83 kg/ha)
- KCl—2 kg/sao (55 kg/ha), applied 10 days after transplanting
- superphosphate—15 kg/sao (417 kg/ha)

Seedling production: Separate seedling nursery bays were used for each level of biofertiliser. Seeds were inoculated with peat cultures containing 25% of final bacterial numbers for the field experiment before sowing nursery plots.

Full rates of biofertiliser were added at transplanting.
Sampling times and variables to be measured:

a. At transplanting
   - microbiological counts of biofertiliser
   - the number of cfu of each strain/g carrier
   - soil samples for total N determination (before fertiliser is applied)
   - seedlings; examine seedlings for both shoot and root growth 12 days after sowing.

b. At harvest
   - tiller weight of each of 10 samples of 45 hills
   - number of panicles/hill in each of 10 hills to be counted per plot and recorded separately
   - number of fertilised seeds/panicle in each of 10 panicles to be counted per plot and recorded separately
   - number of unfertilised seeds/panicle in each of 10 panicles to be counted per plot and recorded separately
   - weight of 1,000 seeds as harvested
   - weight of 1,000 seeds after drying in hot air oven
   - estimated moisture content of the grain
   - grain yield from 10 × 45 hills from each plot. Record separately. Correct to 12% moisture
   - N content of seed to be determined on a sample from each plot.

c. Data analysis by analysis of variance for a split-plot design (e.g. see Steel and Torrey 1980).

Field plan for two replicates

Replicate 1												
BioGro (kg/sao)	2	0	8	4	8	2	4	0	4	0	8	2
FYM (kg/sao)	200	200	200	200	400	400	400	400	300	300	300	300
BioGro (kg/sao)	2	8	4	0	2	0	4	8	4	2	0	8
FYM (kg/sao)	400	400	400	400	200	200	200	200	300	300	300	300
Replicate 2												

Results

This experiment was conducted at Da Moi (Nguyen et al. 2003) and showed that the level of FYM had no significant effect on rice yield. The application of biofertiliser, however, did have a significant effect. The lowest level sufficient to give a maximum effect was 55.5 kg/ha, indicating that BioGro (containing 1N, 3C and 4P) contained satisfactory numbers of viable plant growth-promoting organisms.

The most convincing evidence of the biofertilisers' effectiveness is increased grain yield. Farmers can easily conduct such trials by including a small proportion of strip trials using the following treatments:

- biofertiliser with 50% reduced fertiliser application
- 100% normal practice fertiliser application with no biofertiliser
- 50% normal practice fertiliser application with no biofertiliser.

The first two treatments allow the farmer to confirm that biofertiliser has improved the efficiency of nutrient use or that full application of chemical fertiliser is unnecessary. The third treatment confirms that the biofertiliser has a response.

Appendixes

1 BioGro quality control report template

A BioGro quality control report example template is reproduced below. Table 1 of the results section of the report lists the results of plate counts and can be sent as a preliminary report. Table 2 of the results section lists results of confirmatory tests and can be sent as a follow up report to confirm original rating. Tests on moisture content of inoculants may also be included to indicate confidence in shelf life.

BioGro QC Report

Sample ID

Source of BioGro	
Date of manufacture	
Arrival date	
Number of packet	
Number of sample	
Date of field application	
Place of field application	
Date of testing	
Analyser	
Test method performed	

Results

Plate counting

Sample number	Selective media	Number of bacteria	Notes

Direct ELISA (using specific IgG conjugate)

Sample number	Dilution series	50% maximum value	Notes

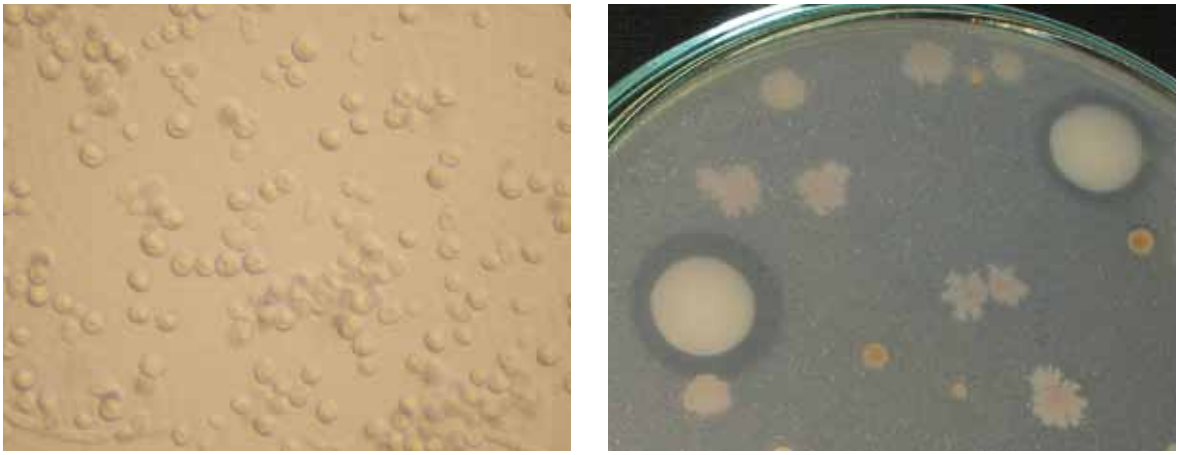
2 Examples of actual reports from the quality control of BioGro in Vietnam

Source of BioGro	Biofertilizer Action Research Centre (Hanoi University of Science)
Date of manufacture	
Arrival date	Nov 15 – 2009
Number of sample	2
Place of field application	Binh Liem – Bac Binh District - Binh Thuan Province Binh Le – Bac Binh District - Binh Thuan Province Nong Truong Co Do
Date of testing	Nov 17 – 2009
Analyser	Tran Thi Kim Cuc
Test method performed	Plate counting by selective media

Results

Plate counting

Selective media	Number of bacteria		Notes
	Sample 1	Sample 2	
NA + tetracyline (HY determination)	B9: 2.20 × 10^6 E19: 1.00 × 10^6 HY: 0.9 × 10^6	B9: 1.20 × 10^6 E19: 1.00 × 10^6 HY: 0.7 × 10^6	
NA + cycloheximide and vancomycin (1N determination)	No result	No result	
MNA (heat selection at 80 °C)	B9: 1.60 × 10^6	B9: 0.9 × 10^6	
MNA	B9: 1.30 × 10^6 E19: 1.30 × 10^6	B9: 1.40 × 10^6 E19: 1.30 × 10^6	
Pikovskaya (P-solubilised bacteria)	HY: 1.50 × 10^6	HY: 1.50 × 10^6	
Tryptic soy broth + agar	B9: 1.10 × 10^6 E19: 1.00 ×x 10^6 1N: no result	B9: 1.50 × 10^6 E19: 1.20 × 10^6 1N: no result	



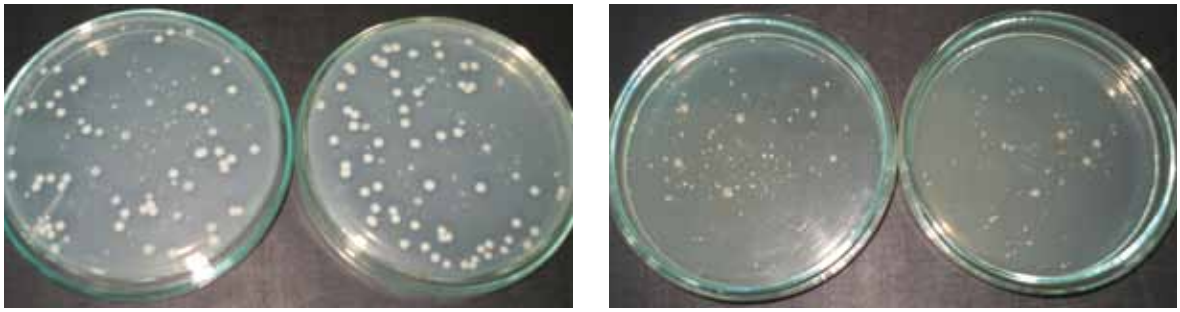
The morphology of HY under microscope (left) and on Pikovskaya media (right)

Source of BioGro	IAS
Date of manufacture	April 15 – 2010
Number of sample	4
Place of field application	Cai Lay – Tien Giang Province Hau Giang Province Binh Dinh
Date of testing	April 19 – 2010
Analyser	Tran Thi Kim Cuc, Tran Minh Hien
Test method performed	Plate counting by selective media

Results

Plate counting

Sample number	Selective media	Number of bacteria	Notes
HY	NA + tetracycine	1.15×10^7	
	Pikovskaya (P-solubilised bacteria)	1.14×10^7	
1N	NA + cycloheximide and vancomycin	3.90×10^7	
B9	MNA (heat selection at 80 °C)	8.50×10^6	
E19	MNA(heat selection at 80 °C)	8.00×10^6	



HY colony on Pikovskaya media (left) and 1N colony on NA (cycloheximide and vancomycin) (right)

3 Data checklist for field experiments

The table below lists all the data that should be collected during field experiments.

Pre-planting data (block information)		Details
Location	Province	
	Village	
	Coordinates	
	Farm/farmer name	
Site conditions	Soil type/texture/cation exchange capacity/% organic carbon	
	Elevation, slope, topography	
	Irrigation method	
	Plot size	
	Site history (e.g. previous crop)	
Soil characteristics (5–10 cm depth)	Soil pH	
	Available nitrogen	
	Available phosphorus	
	Exchangeable potassium	
	% organic carbon	
	Soil temperature	
	Water content	
Soil characteristics (> 30 cm depth)	Soil pH	
	Available nitrogen	
	% organic carbon	
Planting details	Year	
	Date	
	Method (seeding, transplanting)	
	Cultivar	
	Geometry (plants/m^2)	

Fertilisers (treatment information)		Details
Nitrogen	Type	
	Application 1 (date + rate)	
	Application 2 (date + rate)	
	Application 3 (date + rate)	
	Total application rate (kg/ha)	
Phosphorus	Type	
	Application 1 (date + rate)	
	Application 2 (date + rate)	
	Application 3 (date + rate)	
	Total application rate (kg/ha)	
Potassium	Type	
	Application 1 (date + rate)	
	Application 2 (date + rate)	
	Application 3 (date + rate)	
	Total application rate (kg/ha)	
Farmyard manure	Type	
	Application 1 (date + rate)	
	Application 2 (date + rate)	
	Application 3 (date + rate)	
	Total application rate (kg/ha)	
Biofertiliser	Type	
	Application 1 (date + rate)	
	Application 2 (date + rate)	
	Application 3 (date + rate)	
	Total application rate (kg/ha)	
Biofertiliser characteristics	Strain combination	
	Carrier details (type, pH, % carbon, etc.)	
	Date of manufacture	
	Quality control (yes/no, cell numbers)	
	Application method	

Harvest data (response information)		Details
General	Date of sampling	
	Days after sowing	
	Number of replicates	
Plant characteristics	Plant dry weight (per m^2)	
	Number of tillers (per m^2)	
	Number of panicles (per m^2)	
	Nitrogen content	
	Phosphorus content	
Grain characteristics	Grain dry weight (per m^2)	
	1,000-seed weight	
	Moisture content	
	% protein	
	Nitrogen content	
	Phosphorus content	
Soil analysis (5–10 cm depth)	Soil pH	
	Available nitrogen	
	Available phosphorus	
	Exchangeable potassium	

4 Example of field testing protocols: optimising BioGro use

In the World Bank Development Marketplace project 'Sustaining nitrogen-efficient rice production', approximately 500 farmers in the Mekong Delta region, Vietnam, participated in workshops, with 10% of farmers conducting field experiments. These farmers were required to plant a control plot with regular practice nitrogen fertiliser rates, and a BioGro treated plot at reduced nitrogen fertiliser rates. The farms were located in several provinces selected on the basis of location, farm history and consent, with a view to optimise BioGro use.

At these sites, before planting and fertilisation, two composite soil samples (three subsamples combined for each plot) at 0–10 cm depth were taken for routine characterisation. Analyses included available nitrogen, available phosphorus, cation exchange capacity (CEC), texture, pH, % organic carbon and exchangeable potassium. An extra two composite soil samples from 50 cm depth at each site were analysed for subsoil nitrogen that may be present from earlier rice-growing seasons.

All agronomic inputs throughout the growing season were recorded by farmers, including fertiliser inputs, pesticide applications and irrigations, as instructed by survey forms distributed at recruitment and training sessions. Radiation and rainfall data were obtained from the Vietnamese Bureau of Meteorology and supplemented with farmer records. The difference in plant photosynthetic capacity between treated and untreated plots was assessed monthly throughout the growing season at one site, using a portable chlorophyll fluorometer. At harvest, straw and grain yield were measured, together with triplicate analyses of total straw and total grain nitrogen and phosphorus.

In total, 80 soil samples, 60 grain samples and 60 straw samples were analysed in the first season. An additional 100 soil samples, 75 grain samples and 75 straw samples were also analysed in the second season. The data were analysed using correspondence analysis, which is an exploratory technique designed to analyse correspondence between rows and columns of independent and dependent variables. As opposed to traditional hypothesis testing designed to verify existing hypotheses about relations between variables, exploratory data analysis is used to identify systematic relations between variables when there are no (or rather incomplete) expectations as to the nature of those relations. The dependent variables included the difference in grain yield, straw yield, grain phosphorus and nitrogen content and straw at each site, and the independent variables included individual soil and climate characteristics. This allowed determination of specific soil factors as a cause of spatial variation in BioGro effectiveness within seasons, and broader climate factors causing temporal variation in BioGro effectiveness between seasons.

A number of experiments examined the reliability and performance of different types and rates of carrier materials. First, a number of potential carrier materials were identified as low cost or waste organic by-products from primary industries. These included sugarcane waste, aquaculture mud, coconut husk, rice straw and other by-products. All potential carriers were analysed for available nitrogen, available phosphorus, organic matter content, pH and total microbial activity. Each carrier was inoculated with BioGro strains and their survival and activity monitored. Based on results from these experiments, the best carriers have been selected for glasshouse trials with rice.

Glossary

aerotaxic	The movement of a (micro-)organism towards air, usually to access oxygen for respiration.
antibody	A large protein produced by the immune system of higher organisms that can recognise, and bind to, foreign particles, such as micro-organisms.
antisera	The fraction of the blood that contains antibodies.
cross-contamination	The contamination of a pure culture or defined product with an unwanted micro-organism.
ELISA	Enzyme-linked immunosorbent assay; a quantitative method of measurement based on the affinity of an antigen and an antibody, in which the antibody is covalently linked to an enzyme that can produce a colour reaction.
endophytic	Organisms that live at least part of their life within the tissue or cells of a plant.
exopolymer	A linked chain of molecules (usually sugars) that is excreted from a microbial or plant cell, often to aid in adhesion and water retention.
gnotobiotic	Conditions in which a plant (or animal) is raised under aseptic (microbe-free) conditions, followed by inoculation with only specific micro-organisms.
immunoblotting	The blotting of antigens onto a sorbent paper, which can subsequently be probed with specific antibodies.
inoculant biofertiliser	A product that contains selected micro-organisms, often within an inert carrier, which can promote the growth of plants.
lysis	The process in which a cell breaks open.
metagenomic	A description of processes that involve all the (microbial) DNA acquired from an environment, rather than just those processes involving only culturable or known (micro-)organisms.
mutualism	The interaction of two (or more) organisms that results in benefits to both (or all) organisms.
mycorrhiza	A symbiotic relationship between a fungus and a plant, via the root system, which is generally mutualistic.

PCR	Polymerase chain reaction; a method used to produce numerous copies of the original fragments of DNA present in a sample.
pellicle	A thin layer formed by free-living, nitrogen-fixing micro-organisms.
phytohormone	A biochemical compound that affects the growth of plants in a systematic fashion.
phytopathogen	A micro-organism that is capable of infecting a plant, thereby causing disease.
rhizobia	A group of soil bacteria that fix nitrogen inside root nodules of leguminous plants.
rhizosphere	The root zone of a plant, including abiotic components such as soil, solutes, water and gases, and biotic components, such as root hairs, nematodes, fungi and bacteria, along with biologically excreted products.
sao	A measurement unit of area used in Vietnam; equal to approximately 360 m^2 (although it is variable from region to region).
siderophore	A biologically produced chemical (ligand) that can strongly bind to iron.
strain	A subtype of a microbial species containing small differences in genetic material.
supernatant	The liquid fraction resulting from centrifugation.
synergism	Two or more organisms working together to produce a result that cannot be obtained independently.

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