

## Milestone report

# Training manual for product screening and inspection

### Project

Institutionalization of quality assurance mechanism and dissemination of top quality commercial products to increase crop yields and improve food security of smallholder farmers in sub-Saharan Africa – COMPRO-II

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**Date published: June 2016**



COMPRO-II is a project funded by The Bill & Melinda Gates Foundation by a grant to the International Institute of Tropical Agriculture (IITA). IITA works with various partners from the public and the private sectors in Ethiopia, Ghana, Kenya, Nigeria, Tanzania and Uganda for the implementation of the project activities.

**Project Website:** [www.compro2.org](http://www.compro2.org)

If you want to cite this milestone report, please cite it as follows:

Masso, C., AbdelGadir, H. A., Thuita, M. et al. (2016). Training manual for product screening and inspection . [www.compro2.org](http://www.compro2.org) [Date accessed].

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The COMPRO-II project including this milestone has been funded by the Bill & Melinda Gates Foundation through a grant to the International Institute of Tropical Agriculture (IITA). The content of this milestone does not represent the official position of Bill & Melinda Gates Foundation, IITA, or any other organization involved in the implementation of the project. It has been developed based on the best knowledge of the authors and inputs of partners. The readers thereof use, or refer to the information at their own risk and liability.

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## **1 Introduction**

In COMPRO-I (2009-2011), over 100 commercial agricultural products, including microbial inoculants, bio-fertilizers and chemical agents (such as seed and foliar applied fertilizers) were evaluated for quality and efficacy in Kenya, Nigeria, and Ethiopia. Results showed that only few products were of high quality and had a potential economic benefit to farmers. Hence, further screening was recommended, not only to ensure that farmers get the value of their investment, but also to ensure that effective products are used to increase crop yields, improved food security, and generate wealth for smallholder farmers in sub-Saharan Africa (SSA).

This milestone report falls under Objective 2 of COMPRO-II, i.e. continued screening and evaluation of new products. The focus of this training manual is mainly on quality control of bio-fertilizers and bio-pesticides, efficacy testing, and label verification. It includes (i) technical staff in the laboratories for quality control, (ii) regulatory officers who have to ensure that products are properly labelled for consumer protection, efficacious for the intended purpose, and safe to plant, animal, human, and the environment when used as directed, (iii) inspectors involved in the marketplace monitoring for the enforcement of the regulatory framework, and (iv) scientists interested in in-depth assessment of bio-fertilizers and bio-pesticides including molecular techniques.

### **1.1 Purpose and scope of the training manual**

To ensure that staff involved in product screening receive adequate training for efficient and effective screening or inspection of commercial products. After the training, the staff should be able to correctly verify the quality, efficacy, labelling, and safety of the products. The training manual therefore focuses on (i) aspects of laboratory analyses for quality control, (ii) label verification for consumer protection, (iii) efficacy testing to verify label claims or benefit to the end users, and (iv) safety assessment to ensure that plant, animal, human, and the environment are not negatively affected by any commercial product when used as directed.

### **1.2 Justification**

Continuous screening of products in sub-Saharan Africa has been justified based on the following facts:

- Sub-Saharan Africa has been found to be inundated by ineffective products that promise miraculous yields. In some cases, companies misuse environmental arguments for organic, low input farming to promote their products, while farming conditions require substantial nutrient additions for increased crop yields.
- During the first phase (2009-2011), over 100 products were evaluated, but only very few were proven to have consistent positive effects on yield.
- Smallholder farmers use limited financial resources to purchase products, and if they purchase and use ineffective products in crop production, they won't get any positive economic returns. This generally will discourage them, and consequently creates disinterest in new agricultural technologies.

- Quality control procedures are virtually non-existent and/or non-functional, while the quality and contamination in biological products is often variable.

The success of product screening requires adequate methodology. To ensure consistency and accuracy, adequate training is important to strengthen the capacity of stakeholders involved in the process.

### 1.3 Expected outcome

The relevant regulatory bodies will make the training mandatory for staff or institutions involved in quality and label verification, as well as efficacy testing. Equally important, for sustainability, the existing customer-paid service is expected to be streamlined for product screening to ensure that quality, efficacy, and safety are adequately maintained in the full commercialization chain as a result of the training.

### 1.4 Application of the training manual

The training manual targets a diverse audience. Hence, some of the sections may not be relevant for specific audience. Selection of content to use in particular training will be based on the intention of the training. For instance, for laboratories approved by the regulatory bodies to conduct the quality verification for bio-fertilizers and bio-pesticides, the quality control SOPs will be the main focus of the training. Similarly, for institutions mandated to conduct efficacy testing the registration guidelines will be considered. In the case of inspectors, the manual for marketplace monitoring will be used. Finally, research scientists will not only be interested in the quality control SOPs and the guidelines for efficacy testing, but also the molecular techniques.

## 2 Quality verification in the laboratory

Based on the COMPRO-II framework for product screening, bio-fertilizers and bio-pesticides intended to the marketplace in the project countries must first undergo quality control in the laboratory before there are taken for further assessment in the greenhouse and/or field conditions. Six standard operating procedures (SOPs) have been developed for five types of bio-fertilizers (i.e. rhizobia, arbuscular mycorrhizae fungi, *azotobacter*, *azospirillum*, and phosphorus solubilising bacteria) and one type of bio-pesticide (i.e. *trichoderma*) (Annexes 1-6).

Since the market is quite dynamic, it has been recommended that when the regulatory bodies do not have SOPs for specific bio-fertilizers or bio-pesticides such as consortium products, the product proponents must provide the regulatory bodies with the SOP that the manufacturer is using for quality assurance and quality control. The SOP will then be cross-validated by the regulatory bodies before adoption. Such an approach is already being used by the Tropical Pesticide Research Institute (TPRI) in Tanzania. The SOPs are generally organized as follows:

- ✓ The purpose
- ✓ The minimum criteria for quality control
- ✓ The recommended test method for quality verification

### **3 Label verification, efficacy testing, and safety assessment**

More details are included in the country-specific registration guidelines presented under the report of Objective-3's milestone-3 (i.e. established functional legislations and regulatory systems; [www.compro2.org](http://www.compro2.org)). The Tanzanian registration guideline is attached as an example (Annex 7). Briefly, label verification is to ensure that the label of a given product meets the regulatory requirements in terms of mandatory information. It is meant to ensure that products are not misrepresented, and labels do not contain misleading information. Stated differently, label verification will ensure that farmers get the most accurate information about the active ingredients and the expected benefits of a given product when used as directed. The section on labelling includes a detailed list of specific requirements.

Efficacy testing is to generate evidence about the performance of the product when used in the environment where it is intended for use. The regulatory bodies have established the requirements for testing commercial products for registration purpose; these have been included in the regulatory frameworks. The section on efficacy mainly includes the following items:

- ✓ Good quality system procedures
- ✓ Requirement on selection of experimental sites for efficacy testing
- ✓ Minimum number of trials required
- ✓ The spatial and temporal distribution of the trials
- ✓ Specific requirements for bio-fertilizers recommended for several crops
- ✓ Trial design
- ✓ Preventing cross-contamination between experimental plots
- ✓ Treatment structure
- ✓ Planting window
- ✓ Equivalence of data
- ✓ Expiry date and shelf life for bio-fertilizers
- ✓ Measurable parameters
- ✓ Data analysis
- ✓ Profitability analysis

Equally important, guidelines for safety assessment have been recommended. Most of them are not only included in the registration guidelines, but also in the application form for registration. Evidence to support the safety of a given product shall be attached to the application form. In the section of safety, the requirements to substantiate the safety of a bio-fertilizer are outlined.

### **4 Inspection**

One of the issues related to the quality control of bio-fertilizers and bio-pesticides is the lack of adequate marketplace monitoring frameworks. The Training manual for inspectors (Annex

8) is meant to outline the responsibilities of inspectors and what they need to understand to effectively perform their duty. The roles and responsibilities of a (bio-) fertilizer inspector are based on the authority of the fertilizers act and regulations, as well as the bio-fertilizers registration guidelines. Hence, inspectors should be very familiar with those tools. The manual mainly includes the following:

- ✓ principles and techniques of (bio-) fertilizer inspection
- ✓ identification and etiquette of the inspectors
- ✓ safety consideration,
- ✓ power of inspectors
- ✓ responsibilities of a (bio-) fertilizer inspector
- ✓ label review
- ✓ sampling requirements

## 5 Good quality control systems and molecular techniques

The content on molecular techniques (Annex 9) explains the various aspects of post DNA extraction including Polymerase Chain Reaction (PCR), Restriction Length Polymorphism (RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), etc. It also gives guidance on preparation of various growth media types for culturing different microorganisms. The section on mycorrhiza describes the various types of mycorrhiza, identification, infection features etc. the procedures to prepare trap cultures, MPN and characterization of bacteria isolates using tests such as Gram staining, oxidase and catalase tests are also covered. Quality testing for commercial inoculants through counts of Colony Forming Units (CFU) is also covered. Information on the infection and nodule formation by rhizobia, isolation and identification of *Fusarium* and *Trichoderma* are also included. General guidelines on maintenance of contaminant free working area in the lab are also given. Information in this section complements the SOPs recommended by the regulatory bodies. The main sections of Annex 9 include:

- ✓ DNA Extraction
- ✓ Polymerase Chain Reaction (PCR)
- ✓ Electrophoresis
- ✓ Restriction Fragment Length Polymorphism (RFLP)
- ✓ Denaturing Gradient Gel Electrophoresis (DGGE)
- ✓ Selective Growth Media
- ✓ Mycorrhiza
- ✓ Characterization of isolated microorganism
- ✓ Quantification of cells contained in the commercial products
- ✓ Phylogenetic analysis
- ✓ *Rhizobium*
- ✓ *Fusarium*
- ✓ Identification of *Trichoderma* spp.
- ✓ Surface and air control tests



## **6 Conclusions**

Effective application of the training manual will strengthen the capacity of the national systems to (i) verify the quality of products intended for use in a given country across the full commercialization chain, (ii) assess whether the products are properly labelled, (iii) evaluate the performance of the products when used as directed, and (iv) cross-check the safety of the products based on appropriate scientific evidence. Given the recent implementation of the regulatory frameworks for commercial products of interest, adequate training of the target and interested stakeholders is critical to successfully fulfil the mission. The training manual is a dynamic document that will require regular update as novel science arises including new (laboratory) techniques or additional SOPs. The same will apply to the updates of the various registration guidelines.

## **Annex 1: Standard operating procedures for testing the quality of *rhizobium* inoculants**

*Note: The standard operating procedure (SOP) retained by the Tanzania Fertilizer Regulatory Authority (TFRA) has been used in this manual as an example. However, similar SOPs have been developed in Ethiopia, Ghana, Kenya, Nigeria, and Uganda. For training or application, the country-specific SOP will have to be used. However, for countries without their own SOP, they can customize this one. The list of reference includes the various materials used to compile the SOP.*

### **1 Description of selected terminologies used in the standard operation procedure**

**Biofertilizer:** is a substance which contains living microorganisms which are applied to soils, seeds, or plant surfaces to colonize the rhizosphere or the interior of the plant and to promote growth by increasing the supply or availability of nutrients to the host plant.

**Bioenhancers:** Substances that increase the bioavailability of active ingredients, vitamins and plant nutrients.

**Biostimulants:** Living organisms containing strains of specific bacteria, fungi or algae which recycle plant nutrients, enhance nutrients uptake, provide physical barriers against pathogens, stimulate growth, decompose organic residues or act as catalytic agents that promote the healthy growth of plant beneficial soil organisms.

**Bacteria:** A Kingdom of single celled microorganisms whose cells lack membrane-bound nucleus (prokaryotic) and that are ubiquitous living as symbionts, parasites or saprophytes in the environment.

**Symbiosis:** A mutually beneficial relationship between two living organisms.

**Rhizobia:** Are gram-negative bacteria that convert atmospheric nitrogen into ammonia in association with plants after becoming established inside root nodules of legumes.

**Contamination:** presence of other microorganisms other than rhizobia not declared on the label.

**Aseptic Conditions:** An environment where no microorganisms is present. It can be obtained by using a Bunsen burner to create an aseptic zone which is the aspheric area around the

flame with a diameter of approximately 15 cm or a Laminar flow hood with sterile air continuously flowing from inside and out of the hood. The high pressure of air flow from the hood prevents the air from outside to come inside and contaminate the environment in the hood; aseptic conditions are also created by cleaning surface with 70% ethanol.

**Microorganisms contamination:** Every plate, tube, pipette, or other instruments (glassware, pestles, eppendorff tube...) which has been in contact with microorganisms and cannot be sterilized by the flame of a Bunsen burner or any other decontamination procedure is considered as contaminated.

**Contaminated by toxic chemicals:** Every tube, flasks, pipette or other instruments which has been in contact with toxic chemicals is considered contaminated

**Good Laboratory Practices (GLP):** The principle of GLP have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a management concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored recorded and reported. Its principle also includes the protection of human and the environment.

**Mother tube/plate/product:** Tube/plate/product from which the bacteria are picked for inoculation.

**Substrate:** Any material, that serves as source of food/energy for an organism.

**Carrier:** An inert material used as delivery medium for live microorganisms from the production site to the field of application. The carrier is the major portion of inoculant. There is presently no universal carrier or formulation available for release of microorganism into soil. Materials and types of formulations of carriers vary: Slurry, powder, peat, liquid, or clay. A good carrier should have the capacity to deliver the right number of viable cells in good physiological conditions at the right time.

**Growth Medium:** growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms

**Agar:** Agar consists of a mixture of agarose and agaropectin. Agar is used to provide a solid surface containing [medium](#) for the growth of [bacteria](#) and [fungi](#).

**Aseptic :** free of microbial contamination.

## 2 Introduction

Inoculation of legumes with rhizobia has the overall objective of delivering to the plant's rhizosphere the maximum number of appropriate rhizobia at the time of nodule initiation. The delivery of high quality rhizobial inoculants to farmers is one of the fundamental objectives of the Tanzania Fertilizer Regulatory Authority (TFRA). The quality and efficacy of *rhizobium* inoculants are determined by the strain(s), the abundance of cells as well as the formulation. The desired characteristics of inoculants are therefore defined by high strain density, long shelf life, and absence of contaminants, or minimum contamination. Thus, the products must meet or exceed regulatory quality requirements. This document is intended to provide procedures for carrying out requisite tests for inoculant quality assessment.

## 3 Purpose

The Standard Operation Procedure (SOP) is intended to guide laboratories appointed by regulatory agencies in screening of rhizobial products for quality. Based on the information on the product label, the screening is to confirm the strains, populations and ability to produce effective nodules. This is to guarantee customers quality product devoid of contaminants that can reduce the efficacy of the product or are harmful to plants, human, animals and the environment. The final outcome is to guide the regulatory agency on the quality of the product and its suitability for use by farmers. The SOP outlines the procedures provided by the regulatory agency to generate quality data as stipulated on the label and of acceptable standards.

#### 4 Minimum criteria for inoculant quality

In addition to the requirements in the registration guidelines related to labeling, efficacy, and safety, rhizobial inoculants intended for sale in Tanzania shall meet the quality criteria listed below. The Tanzania Fertilizer Regulatory Authority reserves the right to request additional information related to the quality of the product when deemed necessary.

- 1) Viable cell:  $10^{-7}$  cells/g of viable rhizobia.
- 2) Contamination level: no contaminant at  $10^5$  dilution
- 3) pH: 6.0 – 7.5
- 4) Moisture content (per cent by weight of finished product): maximum of 8 – 12% (in case of solid carrier based)
- 5) Particle size: 90% should pass through 250 micron IS sieve for powder formulation (60 BSS - British Sieve Size)

#### 5. Quality tests

Quality tests should include a plate count on yeast extract mannitol agar (YMA) containing Congo red (for viable counts of rhizobia and observation of contaminants) and a plant infection test. Enumeration of contaminants can be achieved through a plate count on peptone glucose agar.

##### 5.1 Viable cell counts (plate count methods)

Viable rhizobial cell numbers in inoculants prepared with sterile carrier can be measured by plate count methods. Most inoculants prepared using non-sterile carrier contain so many fast growing contaminants that plate count procedures are impractical. Plate count methods involve placement of a sample containing viable cells onto the nutrient media surface and counting the resulting colonies after a period of incubation. Since rhizobial inoculants are expected to contain a very large number of viable cells, it is necessary to dilute the sample accurately prior to application to the media so that a countable number of discrete colonies will grow. The most widely used media for counting rhizobia is yeast-extract mannitol agar (YEMA or YMA), often containing Congo red to assist in differentiating contaminants from rhizobia.

##### 5.1.1 Serial dilutions

The first step in counting viable cells in rhizobial inoculant is to prepare a dilution series which will cover the range of expected viable cells. The most commonly used dilution ratio is 1:10 per dilution step. This is equivalent to 10 g (wet weight) of inoculant added to 90 mL sterile diluent (or 11 g inoculant into 99 mL).

The dilution procedure must be a systematic and accurate sub-division of an inoculant. Consequently, transfer volumes must be removed prior to particles settling and the dilutions are shaken prior to transfer onto plates. The bottle for the initial inoculant suspension should accommodate the addition of at least 11 g inoculant to 99 mL (a 1:10 dilution) sterile diluent and still have adequate room for good mixing by shaking or vortexing.

Prepare the dilution series as follows (adapted from Olsen et al. 1996):

Prepare the diluent using sterile phosphate-peptone buffer of the following composition per 1 L distilled water: 1.0 g of Peptone; 0.34 g of  $\text{KH}_2\text{PO}_4$ ; and 1.21 g of  $\text{K}_2\text{HPO}_4$ . The pH of the final preparation should be adjusted to 7.0.

- i. An alternative diluent to phosphate-peptone solution is to use a one-quarter strength solution of the mineral salts present in yeast extract mannitol broth (the yeast extract and mannitol are omitted). Aseptically adjust the pH to 7.0 using 1.0 N NaOH or 1.0 N HCl.
- ii. Mix the inoculant thoroughly within its bag. Aseptically remove the inoculant (e.g. with a flamed and cooled spatula), and weigh out 11 g if using 99 mL dilution blanks (10 g if using 90 mL dilution blanks).
- iii. Suspend the inoculant in sterile diluent and place the bottle on a wrist action shaker for 10 minutes of vigorous mixing. Adjust the bottle horizontally on the shaker so that the suspension is hitting both ends of the bottle on each cycle. This represents the first dilution step ( $10^{-1}$ ).
- iv. Carry out the 10-fold dilution series to the required level. Serially dilute the final 10-fold dilution by pipetting 1-5 mL (transfer volume) into sterile diluent to provide six serial 5-fold dilutions.
- v. The appropriate amount of diluent can be calculated as follows:

Diluent volume = [(dilution ratio - 1) × transfer volume]

For example, for a dilution ratio of 5 and a transfer volume of 2 mL, the diluent volume is  $(5-1) \times 2 = 8$  mL.

### 5.1.2 Plating

Prepare serial 10-fold dilutions through the highest dilution required, and plate only those dilutions that are needed to enumerate cells within the range of interest. For the drop plate method, it is recommended to plate  $10^{-4} - 10^{-7}$ , while the  $10^{-5} - 10^{-7}$  dilutions are sufficient for the spread plate technique. Preparation of the inoculant dilution series is the same for drop or spread plate techniques.

As stated by Olsen et al. (1996), keep in mind that the accuracy of the plate count is dependent upon starting with a representative sample that is accurately diluted. Mix each dilution well and do not allow settling to take place before removing the chosen volume for the next dilution. Use a fresh pipette or pipette tip for each dilution level. Do not let sample dilutions stand for any longer than necessary before plating. Time the work so that it can be completed, from drawing of the sample to putting the plates in the incubator, in a single work session. Until considerable experience has been gained, work with one sample (start to finish) at a time.

#### 5.1.2.1 The drop plate technique

The drop plate technique involves dropping known volumes (usually 20–30  $\mu$ L) of sample dilutions onto YEM agar plates. The drops are not spread, but are allowed to absorb into the agar surface. Resultant colonies are counted from the dilution which produces the largest number (usually about 20) of discreet non-confluent colonies. The plate is usually marked into eight sections to receive eight drops. Four replications of two different 10-fold dilutions can be accommodated on a single plate. The drop plate technique uses agar plates considerably more efficiently than the spread plate method. Experienced users report that more practice is needed to obtain consistent results with the drop plate technique than with the spread plate technique. If, however, a Pasteur pipette (using disposable tips and measuring accurately within the 20–30  $\mu$ L range) is used, the advantages in time and material saving can be considerable.

1. The drop plate procedure is as follows: Prepare appropriate media and plates. It is important that the surfaces of the plates are not "wet." Plates prepared a few days in advance will have the chance to "dry." Mark the plates (on the bottom) into 8 equal pie shaped sections and label them appropriately.
2. From the most dilute of the chosen range of dilutions ( $10^{-7}$  dilution), drop 25  $\mu\text{L}$  of sample about 2.5 cm from the edge of the plates and from a height of about 2 cm. replicate each dilution 4 times (4 drops individually delivered to 4 pie shaped sections).
3. Using a fresh sterile pipette tip and the next least dilute sample suspension ( $10^{-6}$ ), repeat step 2 above, filling the remaining 4 sections of the plate. The plate now has eight drops in total (4 replicate drops from each of two successive dilutions). Do not spread the drops. Leave the plate face up until the sample drops have been completely absorbed into the agar.
4. Repeat steps 2 and 3 above on a second plate, using the other two dilution levels selected for plating ( $10^{-5}$  and  $10^{-4}$  dilutions). Two plates will accommodate 4 dilutions of four replicates each.
5. Allow all sample drops to be absorbed completely, then invert and incubate the plates.
6. Obtain counts from the dilution level drops which show the largest number of discrete colonies free of confluent growth. Count from the "underside" of the plate, marking the position of each colony with a marker as it is counted.
7. Average the counts of the four replicate drops and calculate the number of viable cells in the original sample by accounting for the sample dilution and the volume of the drops used. For example, if the average colony number is 20, the volume of the drops is 25  $\mu\text{L}$ , and the dilution level is  $10^{-5}$  then:  $20 \times 1000/25 \times 10^5$  viable cells per gram.

#### 5.1.2.2 The spread plate technique

The methodology by Olsen et al. (1996) is recommended for this section. The spread plate is the most reliable and commonly used method of plate count enumeration of rhizobia. The method involves spreading a known volume (usually 100  $\mu\text{L}$ ) of a known dilution of sample over the agar solidified surface of a nutrient media. Following incubation for growth, the resulting colonies are counted at a dilution yielding 30–300 colonies per plate. Since plating at each dilution level is normally replicated 3–5 times, the amount of material and time



invested plating and counting can become considerable. The spread plate procedure is as follows:

1. Prepare the required number of YEMA plates and sterile spreaders ("hockey sticks"). The plates should not be "wet" on the surface. Label all plates with a standard nomenclature
2. Prepare 10-fold dilutions of the sample  $10^{-1}$ – $10^{-7}$
3. Transfer 100  $\mu$ L of the highest dilution to be plated ( $10^{-7}$ ) to each of three replicate plates. Have sterile, cool "hockey stick" spreaders ready. A 100  $\mu$ L Pasteur pipette using disposable tips is much better for sample delivery than attempting to hand deliver 0.1 mL volumes from a 1 mL pipette. Use a fresh, sterile pipette tip for each dilution level.
4. Minimize the time that the samples are on the plate before spreading. Spread the sample over the agar surface with a sterile spreader. Move the spreader back and forth in a straight line while rotating the plate on a turntable for approximately 10 seconds. Do not allow the tip of the spreader to deliver sample liquid to the extreme edge of the media where it touches the Petri dish. Do not let the spreader dig into the media.
5. Repeat steps 3 and 4 for the selected number of dilutions to be plated working from the most dilute sample to the least dilute. Use a sterile spreader for each dilution level.
6. Allow the plates to stand right side up until the sample liquid is completely absorbed, then invert the plates for incubation.
7. After an appropriate incubation period, count the colonies on the replicate plates of the dilution showing discreet colonies in the countable range of 30–300. Do not count the plates too early. If the colonies seem to have grown up too fast, they are probably not rhizobia.

Calculate the number of viable cells in the original sample by accounting for all dilutions and for the volume plated. For example, if the average number of colonies from plating 100  $\mu$ L per plate from the  $10^{-5}$  dilution is 62, then  $62 \times 10 \times 10 = 6.2 \times 10^7$  viable cells per gram. Use the mean from the three replicate plates.

### 5.1.3 Reagents for yeast-extract mannitol agar (YMA) media (**adapted from Olsen et al. 1996**)

Table 1. Concentrated stock s for preparation of YMA

1. K <sub>2</sub> HPO <sub>4</sub>	25.0 g /500 mL	6. H <sub>3</sub> BO <sub>3</sub>	50.0 mg /500 mL
2. NaCl	10.0 g /500 mL	7. Zn SO <sub>4</sub>	50.0 mg /500 mL
3. CaSO <sub>4</sub> .2H <sub>2</sub> O)	10.0 g /500 mL	8. CUSO <sub>4</sub> .5H <sub>2</sub> O	25.0 mg /500 mL
4. MgSO <sub>4</sub> .7H <sub>2</sub> O	10.0 g /500 mL	9. MnCl <sub>2</sub> . 4H <sub>2</sub> O	25.0 m g /500 mL
5. Fe-EDTA	1.25 g /25 mL	10. NaMoO <sub>4</sub> .2H <sub>2</sub> O	5.0 mg /500 mL

To prepare YMA, add to 950 mL of purified water 10 mL of stock concentrates 1, 2, 3, 4, 6, and 0.2 mL of stock concentrate 5.

Add mannitol, yeast extract, and if desired, agar, CaCO<sub>3</sub>, and Congo red.

**Table 2.** YMA rhizobial plate-count media (Olsen et al. 1996)

Nutrient	G per litre	Micronutrients	g per litre
K <sub>2</sub> HPO <sub>4</sub>	0.5	H <sub>3</sub> BO <sub>3</sub>	1.0
NaCl	0.2	Zn SO <sub>4</sub>	1.0
CaSO <sub>4</sub> .2H <sub>2</sub> O)	0.1	CUSO <sub>4</sub> .5H <sub>2</sub> O	0.5
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2	MnCl <sub>2</sub> . 4H <sub>2</sub> O	0.5
Mannitol	10.0	NaMoO <sub>4</sub> .2H <sub>2</sub> O	0.1
Yeast extract	2.0	5. Fe-EDTA (sequestrene)	10.0
Agar	18.0		

Adjust pH to 7.0 with 1N HCl. To add Congo red, add 10 mL of a 1:400 (0.1 per 40 mL H<sub>2</sub>O) aqueous solution of dye to the media before autoclaving.

### 5.2 Peptone-glucose test for contamination on plates

Almost all rhizobia grow poorly, if at all, on glucose-peptone media, whereas many potential contaminants grow readily and produce pH changes. Bromthymol blue (BTB) and bromcresol purple (BCP) are pH indicators. If either dye is incorporated in peptone-glucose media it will indicate major pH shifts which are often associated with growth of contaminating organisms, but not with rhizobia (because the rhizobia do not grow). Bromthymol blue turns yellow at pH 6.0, blue at pH 7.6, and is green between pH 6.0 and 7.6. Bromcresol purple turns yellow at

pH 5.2 and purple at pH 6.8. Rapid growth (24 -48 hours) of streaked culture on this media, particularly if associated with colour change (pH reaction), strongly indicates contamination.

#### 5.2.1 Composition of Peptone-glucose media

1. 1000 mL purified water
2. 5 g glucose
3. 10 g peptone
4. 15 g agar
5. 10 mL of 1% bromcresol purple in ethanol (0.10 g BCP in 10 mL ETOH) or
6. 5.0 mL of 0.5% BTB

#### 5.2.2. Plating on peptone glucose agar (PGA)

Enumeration of contaminants on peptone glucose agar is done using the spread plate method. Prepare the required number of PGA plates and prepare 10-fold dilutions of the sample  $10^{-1}$ – $10^{-7}$ . Proceed with plating as explained for the spread plate method. Contaminants should not exceed  $10^6$  per gram of inoculant.

#### 5.3 Enumeration of rhizobia in inoculant using the most probable number (MPN) plant infection assay (Olsen et al. 1996)

The most-probable-number (MPN) technique is a means to estimate microbial population sizes. The technique is widely used to enumerate rhizobia based upon the ability of rhizobia to nodulate appropriate host legume plants. The method relies upon the pattern of positive and negative nodulation responses of host plants inoculated with a consecutive series of dilutions of rhizobia containing sample suspension. The results are used to derive a population estimate based upon the mathematics of Halvorson and Zeigler (1933). MPN enumeration of rhizobia is dependent upon the ability of the researcher to grow the legume hosts in a healthy and replicated fashion, and to keep the plants free from rhizobial contamination for a period of up to 4 weeks.

##### 5.3.1 Designing an MPN assay

The process of designing an MPN assay for legume inoculants involves selecting the degree of initial dilution of the sample, base dilution ratio of the serial dilutions applied to plants, number of serial dilution steps applied to plants, number of replicate plants to be inoculated at each dilution level, and volume of inoculant to be applied to each plant unit. Care must be

taken to design MPN assays for which population estimates can be obtained through MPN tables. Standard MPN tables do not provide estimates for all dilution ratio-replicate combinations. The base dilution ratio and the number of replicate plants per dilution level are used in the calculation of a confidence factor which describes the reliability of the MPN result. The population estimate is multiplied or divided by the confidence factor to establish the upper and lower limits, respectively, of the confidence interval for the population estimate. Decreasing the base dilution ratio or increasing the number of replicate plants per dilution level results in a narrowing of the range of the confidence interval and a greater resolution of the MPN estimate. Conversely, increasing the base dilution ratio or decreasing replication results in broader confidence intervals. Confidence factors associated with different dilution ratios and replicate combinations are presented in Table 3. For two population estimates to be significantly different from one another, the lower limit of the greater population must be higher than the upper limit of the lesser population. Altering the volume of inoculant applied to the root system of the host legume is another option in the design of plant-infection assays. Most published MPN tables assume a 1.0 mL inoculant volume. An MPN population estimate can be obtained from those tables after applying greater or lesser inoculant volumes by using the relationship:

$$\text{Population estimate} = (1 / \text{inoculant volume}) \times \text{tabular estimate}$$

For example, if an inoculant volume of 2 mL is applied to the root system, the population estimate is half that of the tabular MPN estimate. Similarly, if 0.5 mL is applied, the population estimate is twice that of the tabular value. Increased inoculant volumes (greater than 1 ml) are useful when larger plant growth containers are used, or when the researcher wants to lower the range of population detection. Decreased inoculant volumes (less than 1 ml) are useful when host plants are grown in small culture tubes or when the researcher wants to extend the upper range of population detection. Adjustments in the inoculant volume do not affect the confidence factor.

**Table 3.** Factors for calculating the confidence intervals of Most-Probable-Number estimates

	Factor for 95% confident interval at various dilution ratios <sup>1</sup>				
Replication per dilution	2	3	4	5	10

1	4.01	5.75	7.14	8.31	14.45
2	2.67	3.45	4.01	4.47	6.61
3	2.23	2.75	3.11	3.40	4.67
4	2.00	2.40	2.67	2.88	3.80
5	1.86	2.19	2.41	2.58	3.30
6	1.76	2.04	2.23	2.37	2.98
7	1.69	1.94	2.10	2.23	2.74
8	1.63	1.86	2.00	2.11	2.57
9	1.59	1.79	1.93	2.03	2.44
10	1.55	1.74	1.86	1.95	2.33

<sup>1</sup>Population estimates are multiplied and divided by the confidence factors to establish the upper and lower confidence intervals at ( $P = 0.05$ ), respectively. Confidence factors were calculated using MPNES software (Woomer *et al.*, 1990).

### 5.3.2 MPN procedure for inoculant evaluation

The following is a commonly used MPN design for the evaluation of legume inoculant products. Using growth pouches and a suitable supporting rack, prepare 30 pouches (also called growth units) with a suitable number of healthy host plants per pouch and sterile N-free nutrient solution (30 mL for whole pouches, 15 mL per side for split pouches). This makes six sets of 5 growth units.

- i. Taking a representative sample of the inoculant, dilute the sample by successive 10 fold dilutions (in sterile diluent) to a suitable starting point based on the anticipated number of rhizobia in the product. Given an anticipated rhizobial cell number of greater than  $10^9$  per g, a suitable starting point for further dilutions would be a dilution of  $10^{-6}$ .
- ii. From the  $10^{-6}$  dilution make an additional six serial 5-fold dilutions in sterile diluent (the initial dilution is  $10^{-6}$  the base dilution ratio is 5).
- iii. Apply 1.0 mL to each of four replicate growth units from each of the six 5-fold dilutions. Leave the 5th growth unit in each series un-inoculated. Whether one works from the most dilute of the 5-fold dilutions to the least dilute, or vice versa, depends

upon the technique used to apply the inoculant suspension, but in any case work in order, and be consistent.

- iv. Place the growth-unit rack containing the MPN assay in an appropriate growth environment and maintain suitable plant condition by watering with sterile water.
- v. After 3-4 weeks (depending upon the legume host used) examine the plants and record each growth pouch unit as "+" or "-" for nodulation. Even one nodule per growth unit means a "+" score for that unit. Do not count "maybe" nodules. All six negative control units must be free of nodules (if not, discard the test, repeat, and work on technique).
- vi. For each of the six successive 5-fold dilutions a number of pouches between 0 and 4 will have been scored as "+" for nodulation. The number of "+" scores taken in order of increasing dilution will yield a six digit number. A typical result could be: 4, 4, 4, 1, 1, 0.
- vii. Using either the MPNES computer program or an MPN probability table based on six 5-fold dilutions, 4 replicates, and 1.0 mL volume applied per growth unit, determine the population estimate and 95% confidence limits. This estimate refers to the number of rhizobia present in 1 mL of the suspension from which the first 5-fold dilution applied to the plants was made. Multiply the MPN estimate by the reciprocal of the dilution level of the inoculant suspension prior to beginning the 5-fold serial dilutions (in this case  $10^6$ ) to get an estimate for the number of viable rhizobia in the original inoculant. For the example given above the estimate is  $379 \times 10$  rhizobia per g.

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## Annex 2: Standard operating procedure for testing the quality of AMF inoculants

*Note: The standard operating procedure (SOP) retained by the Tanzania Fertilizer Regulatory Authority (TFRA) has been used in this manual as an example. However, similar SOPs have been developed in Ethiopia, Ghana, Kenya, Nigeria, and Uganda. For training or application, the country-specific SOP will have to be used. However, for countries without their own SOP, they can customize this one. The list of reference includes the various materials used to compile the SOP.*

### 1 Description of selected technical terms used in the standard operating procedure

**Biofertilizer:** is a substance which contains living microorganisms which are applied to soils, seeds, or plant surfaces to colonize the rhizosphere or the interior of the plant and to promote growth by increasing the supply or availability of nutrients to the host plant..

**Fungi:** Multicellular organisms, form separate Kingdom from plants, animals, protists and bacteria with cell walls containing chitin. They can be saprophytic (feeding on dead materials); symbiotic (mycorrhiza, lichen) or parasitic (feeding living organisms)

**Symbiosis:** A mutually beneficial relationship between two living organisms.

**Mycorrhiza:** A beneficial symbiosis between fungus and a living plant root. There are two main types of mycorrhiza Ectomycorrhiza (outside the root system of woody plants) and Endomycorrhiza (penetrate root cell walls of cultivated Plants, e.g., arbuscular mycorrhiza)

**Arbuscular mycorrhiza fungi (AMF):** A group of fungi that inhabit roots of most plants forming mutually beneficial relationship where they exchange nutrients and water for plant carbohydrates.

**Arbuscules:** Branched treelike fungal structures within the root cell walls of a host plant, i.e., one of the treelike haustorial organs in in cortex cells. Arbuscules are the sites of nutrient and sugar exchange between plant and fungus.

**Vesicles:** sacs or swollen cells that are globose, ovoid thin-walled storage organs filled with lipids and glycolipids between root cells or within cell walls and may have a function in fungal food storage for survival and reinfection of new roots. Vesicle development often occurs later in the development of AM infection. Vesicles are not formed by *Gigaspora* spp. or *Scutellospora* spp. Those formed by *Acaulospora* spp. are typically thin-walled and irregularly shaped. Root infection by other **non-mycorrhizal endophytes** can be mistaken for AM infection.

**Auxiliary cells:** Spore-like structure or swollen structure produced terminally by extraradical hyphae of the Genus *Gigaspora*, *Pacispora* and *Scutellospora*. Auxiliary cells formed by *Gigaspora* spp. (spiny) or *Scutellospora* spp. (nodulose?) often remain attached to the root by hyphae after staining.



**Spores:** Multinucleate single asexual reproductive cells mainly produced at the tip of a sporogenous hyphae.

**Mycelia (Mycelium):** Are the vegetative parts of the fungus and are thread-like hyphae. They are the bridges that connect the root with the surrounding microenvironments.

**Hyphae (hypha):** The web of tiny filaments/threads that make up the mycelia.

**Coiled hyphae:** Highly branched structures develop throughout the cortical cells, and can also occur in the epidermal cells. Coiled hyphae often develop smaller side branches and may have a similar function to the arbuscules.

**Inoculum potential:** The ability of total infective propagules (spores, mycelia, infected root fragments) to initiate root colonization.

**Infectivity:** The mycorrhizal inoculums ability to infect the roots.

**Contamination:** Microorganisms other than AMF not declared on the label.

## 2 Introduction

Inoculation with arbuscular mycorrhiza fungi (AMF) commercial products in agricultural, forest and degraded landscapes is a common practice. The AMF benefits are mineral nutrition particularly phosphorus, plant water potential under drought stress, protect plant against some pathogens, among others. The quality and efficacy of the inoculant is determined by the strains, abundance of infective propagules and the inoculant formulation. The desired characteristics of inoculants are therefore defined by high propagule density, significantly long shelf life, and absence of, or minimum contamination.

Product quality control assesses whether product is fit for the purpose. The product must meet or exceed customer and regulatory quality requirements.

## 3 Purpose

The Standard Operation Procedure (SOP) is intended to be used as the official method to control the quality of AMF products intended to be sold in Tanzania. Stated differently, based on the information on the product label, the quality control is to confirm the strains, propagule density, contamination level, and ability to colonize. This is to guarantee customers quality product devoid of contaminants so that the product are efficacious for the intended purpose, and safe to plant, animal, human, and the environment.

## 4 Minimum quality criteria for an AMF product

In addition to the requirements in the registration guidelines related to labeling, efficacy, and safety, an AMF product intended for sale in Tanzania shall meet the quality criteria listed below. The Tanzania Fertilizer Regulatory Authority reserves the right to request additional information related to the quality of the product when deemed necessary.

- 1) Total viable propagules/g of product: minimum of 100 propagules/g of finished product]
- 2) Infectivity potential: 80 infection points in test root/g of mycorrhizal inoculum used
- 3) Contamination level: no contaminant at  $10^5$  dilutions
- 4) pH: 6.0 – 7.5

- 5) Moisture content (per cent by weight of finished product): maximum of 8 – 12% (in case of solid carrier based)
- 6) Particle size: 90% should pass through 250 micron IS sieve for powder formulation (60 BSS - British Sieve Size) (

## 5. Quality tests

### 5.1 Arbuscular Mycorrhizal Fungal: Spore Characterization

#### 5.1.1 Minimum materials and equipment for characterization of spores

1. Bench centrifuges
2. Centrifuge test tubes: 50 ml, round bottom tubes
3. Clinical centrifuge tubes: 15 ml
4. Water bottles,
5. Sieves: 2 mm Nalgene
6. 710 um mesh sieves
7. 250 um mesh sieves
8. 38 or 45 um mesh sieves
9. Beakers: 1L, 250 ml and 100 ml
10. Petri dishes, 90 and 50 mm, or watch glass
11. Glass Pasteur pipette (1 ml)
12. Compound microscopes
13. Dissecting microscopes

#### 5.1.2 Preparation of reagents for isolation and diagnosis of spores

Spores are the most important diagnostic parts of the arbuscular mycorrhizal fungi. Most of the common reagents required are for spore extraction (sucrose), mounting of specimens (Polyvinyl Lactophenol Glycerol (PVLG) and Melzers reagent for spore staining.

##### 5.1.2.1 Spore Extraction

227 g sucrose (normal sugar) dissolved in 500 ml water

##### 5.1.2.2 Mounting Reagents

###### 1) Polyvinyl-Lactophenol-Glycerol (PVLG)

PVLG is used to permanently mount whole or broken spores on glass slides. For best results, mounted specimens should be studied 2-3 days after they are mounted to give time for spore contents to clear. Whole spores will change colour, generally darkening to varying degrees, and shrink or collapse with plasmolysis of spore contents. Discrete layers of the spore wall or flexible inner walls of broken spores will, in some instances, swell to varying degrees and appear fused after long storage. PVLG stores well in dark bottles for approximately one year. The following ingredients and quantity are required:

- a) Distilled water 100 ml
- b) Lactic acid 100ml
- c) Glycerol 10 ml
- d) Polyvinyl alcohol 16.6g

### 5.1.2.3 Melzer's Reagent

Melzer's reagent is important in diagnosis of AMF spore morphology showing dextrinoid reactions of spore wall and inner germination walls. In freshly extracted spores, the intensity of the staining reaction mainly depends on the length of carbohydrate chains present in the components. In stored spores, the reactivity of their sub-cellular structures usually decreases or completely disappears. Iodine staining reactions vary from pale pink (weak reaction) to dark red-brown (moderate reaction) to dark reddish-purple (intense dextrinoid reaction).

Mounts are temporary even when a cover-slip is sealed, and often dries out within 1-2 years of storage and, thereby, usually are unsuitable for further studies. More permanent mounts are made by mixing Melzer's reagent with PVLG in a volume ratio of 1:1 (and storing the mixture in a dark bottle). The staining reaction is diminished slightly, but not enough to cause any confusion as to the intensity of the reaction. In structures staining weakly, the colour reaction fades within a year or two of storage. The solution should be stored in a tight capped bottle and is most easily dispensed from some form of a dropping bottle and the mixture should be stored in a dark bottle. The following ingredients and quantity are required:

- a) 5.0 g Potassium Iodide ( KI)
- b) 1.5 g Iodine
- c) 100 g Chloral hydrate
- d) 100 ml Distilled water

### 5.1.3 Methods of preserving spores

#### 5.1.3.1 Sodium azide for voucher specimens

If spores are not to be examined immediately spores should be stored in sodium azide. Spores die in the sodium azide and degrade naturally over time. They then float, contents often darken or lose their integrity (appearing either cloudy or vacant). However, sub cellular structures largely retain their integrity. Other preservative solutions such as FAA (Formalin + Acetic acid + Alcohol) and lactophenol (lactic acid + phenol) have been used extensively in the past, but evidence from type specimens indicates they can cause major changes or degradation of sub-cellular structures of spores. Spores stored in sodium azide or any other preservative turn red-brown to brown within 10 days. Solutions and vials are stored at 4°C as an added precaution to optimize safety of the workplace.

#### 5.1.3.2 Distilled water and sand for voucher specimens

Store washed spores in distilled water at 4°C. The drawback with storing in water is that parasitized spores (often not detectable at the time of extraction) are sources of spread of colonizing fungal saprophytes or actinomycetes to adjacent spores. Store in distilled water or water agar, or on 0.1% MgSO<sub>4</sub>.7H<sub>2</sub>O solidified with gelling gum in Petri dishes and glass vials and stored in the fridge at 4°C.

## 5.2 Diagnosis of specimen and identification of strains

### 5.2.1 Isolation of spores from a clay carrier based commercial product

*Note: In the case of a different carrier material the product proponent/manufacturer should provide the protocol used to isolate the spores in the formulation. Such protocol should be*

*validated by competent regulatory authority in one of its approved laboratories. Once validated, such a protocol will be adapted for future quality control of AMF products with the same carrier material.*

## **Extraction**

1. The finished product unlike soil does not have plant debris as is the case with field soils and pot cultures. Measure the volume or dry weight of the sample. 50 g product samples are recommended for extraction.
2. If possible pre-soak the product in water before processing. This is particularly important if products are dry or high in clay content. For products with carriers of significant clay content, the clay is likely to cause sieve blockage. To prevent blockage of the finer sieve, sieve quickly and tap the base of that sieve to encourage excess water to drain through or soak product in sodium hexametaphosphate to disperse the clay fraction.
3. Mix the product in water in a 1 L plastic beaker, stir thoroughly and decant through 0.710 mm 710  $\mu\text{m}$  and 0.045 mm 45  $\mu\text{m}$  sieves (other sieves may be included in this series e.g. 0.5 mm 500, 0.25 mm 250, 0.1 mm 100  $\mu\text{m}$  sieves, but each collection will need to be examined separately).
4. Use a jet of tap water to wash the spores and finer product particles through the 710  $\mu\text{m}$  sieve. This water will quickly pass-through the 710  $\mu\text{m}$  sieve, but it is difficult to get the water to drain through the 45  $\mu\text{m}$  sieve because of the build-up of fine particles. The two sieves are separated and a jet of water on the surface of the 45  $\mu\text{m}$  sieve permits the water to drain at that spot. Then the two sieves are stacked together again, and the process is repeated until the water washing through the two connecting sieves leaves the bottom sieve colourless. Generally this means the particles in the top sieve are washed 3 times.
5. In case some of the strains declared are sporocarpic or in Gigasporaceae or with other infective propagules (infected root fragments and mycelia), before discarding the content, examine the collection on the 710  $\mu\text{m}$  sieve (and if more sieves are used as well) for sporocarps, auxiliary cells and spores before discarding. Root fragments and mycelia may also be collected from this sieve for staining. **Note:** The water drains slowly through the lower sieve and may overflow from the 45  $\mu\text{m}$  sieve; therefore keep checking visually the height of the water by carefully separating the two sieves; if the water does overflow the lower sieve, spores are lost.
6. A concentration of AMF spores remains in the pellet on the 45  $\mu\text{m}$  sieve. Collect the particles from the 45  $\mu\text{m}$  sieve by back washing them into a beaker using a small stream from a wash bottle. The contents in a beaker are swirled and poured into two or four or more 50 ml centrifuge tubes (1/3 soil and 2/3 tap water up to within 2.5 cm of the top of the tube).
7. Then balance the tubes by weight and thoroughly stir the mixture and then centrifuge for 5 minutes at 1750 rpm. Carefully decant the supernatant without disturbing the pellet at the bottom of the centrifuge tube.
8. Suspend the pellet containing the spores in a 48% sucrose (227 g dissolved in 500 ml water) solution and mixed well. Balance the tubes by weight and mix thoroughly immediately before centrifuging for 15 seconds at 1750 rpm.
9. Immediately after centrifugation, carefully decant the supernatant of sucrose solution that contains the spores through a small 45 $\mu\text{m}$  sieve. Immediately rinse the spores retained on the sieve thoroughly with tap water to wash out the sucrose.

10. Wash the AMF spores into a beaker of water using a wash water bottle.
11. Transfer the spores from the 45 µm sieve into a small Petri dish (5cm diameter) with grid lines for examination under a dissecting microscope at x40.
12. Using a dissecting microscope, pick morphologically similar spores, aggregates, and sporocarps by means of of fine glass Pasteur pipette or needle and enumerate and mount on slides for further diagnosis. **Note:** Fine glass pipettes: soften tip of disposable glass Pasteur pipette (1 ml) with flame of Bunsen burner and sharpen by pulling the malleable (soft) tip with a forceps and break the tip to make various sizes of tips to fit different sizes of spores. A light fine tweezers (forceps) is preferably the most convenient with good handling.
13. Spores may immediately be mounted and characterized for identification or stored to consult an expert for diagnosis.
14. Characterization and identification is by a **compound microscope**: biological compound microscope preferably with a Nomarsky's DIC illuminator system. The morphology of spores is the basis of identification. They have characteristic shapes and colour. It is, however, recommended that those who have not yet observed spores should learn from experts how the spores look like. Use pictures in text books and websites to recognize AMF fungal spores.
15. Spores are placed in watch glass or small Petri dish and their shape, colour and the attachment to spore observed.
16. Spores are classified into spore types based on morphology
17. For colour description, a standard soil colour chart or colour chart of Glomelean fungi (INVAM) or colour chart of fungi should be used. The colour chart should be under the same illumination as used for spore observation because colour itself is greatly affected by the characteristics of illumination.

### 5.2.2 Procedures for preparation of diagnostic slides, mounting of slides and observations of spores

Voucher specimens of permanent slides (slides sealed with colourless nail varnish at 5-10 days after mounting) are prepared as reference material.

- 1) Mount spores on slides with PVLG on glass slide. Several slides are made: intact spores mounted on water (to be observed immediately for colour), intact spores mounted on PVLG, crushed spores mounted on PVLG, crushed spores mounted on PVLG containing Melzers reagent. For spores 40 – 50 spores should be examined in PVLG.
- 2) Mounting slides and observations of spores
  - a. Voucher specimens of permanent slides (slides sealed with colourless nail varnish at 5-10 days after mounting) are prepared as reference material.
  - b. Prepare three slides: On the first one, place two drops of water at opposite ends of a microscope slide. On the second, place one drop of PVLG on each end and on the third one, put PVLG + Melzers (ratio 1:1) on both ends. (Slides frosted at one end are preferred for labelling purposes but in case they are not available, a sticker may be used).
  - c. Pick carefully spores with a fine forceps or micro-pipette and place in each drop.

- d. Lower a cover-slip, preferably circular with diameter of 13 mm onto each drop of mounting reagent + spore.
  - e. For each slide, observe the spore as a whole for diagnosis of appearance on one end, and on the other end, crush the spore gently and severely. A needle or pin may be used to crush the spores. Caution must be taken not to break the cover-slip.
  - f. In case of bubbles, gently place ethanol through the space between slide and cover-slip to clear bubble, absorb ethanol with tissue and then gently place the mounting reagent again.
  - g. Slides are properly labelled to indicate the sample identity, mounting reagent and date and link slide specimen with information in database on site of collection, soil type, collector name, institution and spore morphological characteristics.
  - h. For description of spores refer to INVAM WEBSITE (<http://invam.wvu.edu/the-fungi>)
  - i.
- 3) Morphological characteristics are recorded to identify genus of the target fungus. The genus *Archeospora* needs both morphological characteristics and sequence data.
  - 4) Species identification is undertaken with comparison with species description in original references. Species description and pictures are available in INVAM website. To completely verify a species, consultation with experts in AM fungal taxonomy is necessary.

### 5.3 Procedures for spore storage for reference

1. **Sterilization and storage of spores:** Concentrate the spores on the 45µm sieve and then with a squeeze bottle, wash the spores into the sterilization solution. Soak the spores in this solution for 20 minutes. Pour the spores onto the filter which is sticking on the funnel, and rinse with distilled water 5 times. Make a hole in the filter paper and wash the spores into a 100 ml graduated cylinder. Add more water until the volume is 100 ml. Samples can be removed to determine the spore number (Tang, 1986).
2. **Short term storage:** Store the isolated, non-sterilized spores for 2 days in the refrigerator in a beaker of water sealed with parafilm.
3. **Long term storage:** Fill a clean Petri dish 3/4 full of clean, dry sand and pour the sterilized and rinsed spore suspension over the sand. Allow the water to evaporate from the sand by leaving the cover off. This may take overnight to several days. When the sand is dry, cover the Petri dish and seal it with parafilm. Store the sealed and labelled dish at 4°C until the spores are used.
4. **Retrieval of long term stored spores:** Pour the dry sand/spore mixture onto the 250 µm sieve (top), which has been stacked on the 45 µm sieve (bottom). Using distilled water, rinse
5. the spores through the top sieve and collect them from the bottom one (45 µm) with a squeeze bottle containing distilled water. The spores can be washed into a Petri dish.
6. Isolated spores may be identified and/or used to inoculate other plants.

### 5.4 Estimation and quantification of total spore abundance and infective propagules

- 1) Total viable spore counts under a dissecting microscope from 50 g sample of product. Depending on the carrier material, spore isolation methods may vary.

- 2) There are more than spores in the products that serve as infective propagules. Other propagules are infected root fragments and mycelia. Enumerating spores abundance may therefore underestimate or overestimate the ability of AMF to colonize. Other methods such as the Most Probable Number (MPN) method and Mycorrhizal Product Infectivity (MSI) test have been used to detect viable infective propagules. The method is used for enumeration of AMF viable infective propagules in the product. The infective propagules include spores, hyphae and infected root fragments. The procedure described here is for four fold dilution. Other dilutions such as two fold or tenfold may be used depending on the inoculum potential of products, the former for low inoculum potential and the latter for high inoculum potential
- 3) Materials
  - a) Plant tubes (150cm x 2.5 cm)
  - b) Stands for keeping plant tubes/Polythene bags (30cm x 20cm)
  - c) Sterilized sand: product (1:1) mix
  - d) Physical balance
  - e) **Plant material:** Plant is chosen depending on its mycorrhizal colonization potential and on the aim of the study. Onion seed.
  - f) **Products:** Product is serially diluted in sand previously sterilized.
- 4) Dilution: example of 4 fold dilutions
  - a) Five replicates
  - b) Start with 65 g of finished product per each pot.
  - c) Dilute product in sand:  $4^0$ ,  $4^{-1}$ ,  $4^{-2}$ ,  $4^{-3}$ ,  $4^{-4}$
  - d) Dilution for  $4^0$  stands for the undiluted product.
  - e) To prepare the  $4^{-1}$  dilution for example, mix 65 g of the test product (undiluted) with 185 g of sterile sand (autoclaved) = 250 g; mix thoroughly..
  - f) To prepare the subsequent dilution, follow the same approach, but using the immediately precedent dilution. For instance, to prepare the  $4^{-2}$  dilution, mix 65 g of Dilution 4-1 material with 185 g of sterile sand (autoclaved) = 250 g; mix thoroughly.
  - g) Place 50 g of each replicate and dilution in the experimental pots.
  - h) Calculate dry weight of the test product per fresh weight used to give the final propagule number in gram of dry product (72 h at 70°C).
  - i) Sow 5 pre-germinated seeds (surface sterilized) per pot.
  - j) Grow plants in greenhouse or growth chamber for six weeks.
  - k) Stain the root systems and assess presence or absence of colonization under a stereoscope and check, if necessary, colonization under the compound microscope (mount roots on a glass slide).
  - l) For each of the 5 replicates in each of the four dilutions ( $4^{-1}$ ,  $4^{-2}$ ,  $4^{-3}$ , and  $4^{-4}$ ), one might obtain a combination of numbers such as 5, 5, 3, 2. This means that all 5 replicate tubes are positive for AM colonization in dilutions  $4^{-1}$  and  $4^{-2}$ , three are positive pots tubes in dilution  $4^{-3}$ , and 2 positive pots in dilution  $4^{-4}$ .
  - m) For the calculation of MPN of propagules only three numbers of the given combination are required. The first number (N1) is that corresponding to the least dilution which all the (or the greatest number of) tubes are positive for AMF colonization. The two other numbers (N2 and N3) are those corresponding to the next two higher dilutions. In the example above it would be the combination

N1	N2	N3
5	3	2

- n) The most probable number of AM propagules can then be calculated using MPN table. Make use of the table with these values N1, N2 and N3; the value given for the combination 5, 3, 2 is 1.4. To obtain the MPN of infective propagules of AM fungi in the sample the table value has to be multiplied by the middle dilution i.e.  $1.4 \times 4^3$  Infective Propagules/g finished product  
[http://www.ctahr.hawaii.edu/oc/freepubs/pdf/amf\\_manual.pdf](http://www.ctahr.hawaii.edu/oc/freepubs/pdf/amf_manual.pdf) [Habte and Osorio (2001); Assessed 25 January 2015]

5) Illustration of MPN calculation

If using 50 g per pot, set out the table of results like this:

Dilution	g of product per dilution from test product	Infected reps per dilution
$4^0$	50	5
$4^{-1}$	12.5	5
$4^{-2}$	3.13	4
$4^{-3}$	0.78	3
$4^{-4}$	0.20	2
$4^{-5}$	0.05	1
$4^{-6}$	0.01	0
$4^{-7}$	0.00	0
$4^{-8}$	0.00	0

Formula used:

$$\log \lambda = (x \log a) - K$$

$\lambda$  = number of infective propagules

= mean number of plants infected

= number of total infected reps

Number of reps per dilution

$$= 20 = 4$$

y = number of dilution (s) = s -

$$= 9 - 4$$

$$= 5$$



a = dilution factor

K = Constant found from tables of Fisher & Yates (1970) for the 4-fold dilution series using (x) or (y) for the number of dilutions (s).

N.B. Where neither (x) nor (y) has a value in the table use the (\*) value indicated.

In this example it is the \* value = 0.552

$$\text{Log } \lambda = s \lambda \times z$$

$$\sqrt{n}$$

$\lambda$  = no. of infective propagules

$s \lambda = \sqrt{0.201}$  for 4-fold dilutions

n = no. of reps per dilution

z = tabulated values of probability

90% = z=1.282

95% = z= 1.645 – this is the level that is normally used

99% =z= 2.326

Therefore in this example,

$$\text{Log C.L.} = 1.86 + \sqrt{0.201} \times 1.645$$

$$\sqrt{5}$$

$$= 1.86 + 0.329$$

Therefore log  $\lambda$  upper limit = 2.19

Log  $\lambda$  lower limit = 1.53

$\lambda$  upper limit = 8.9

$\lambda$  lower limit = 4.6

### Summary

So in this example the number of infective propagules ( $\lambda = 6.4$ ) in 50 g F.W. Products falls between 8.9 and 4.6 in 95% of cases [quote as dry weight (D.W) of product].

**Points to note:** *The last dilution must contain no infection and the dilutions must be well mixed.*

## B.6 Infectivity assessment: Methods in Root Analysis for AMF

### B.6.1 Assessment of infectivity potential of spores/propagules

The methodology recommended by Bhattacharyya and Tandom (2012) is recommended here. Briefly, the bioassay is used to determine the number of infective propagules present in the

product. Once the infective propagules (spores, mycelia, and vesicles in the root fragments) come in contact with the host roots they give out a turgid mycelial structure (the appressoria), which is the initial step in root penetration. The entry point can be visualized by staining and enumerated as a measure of the infectivity of the inoculum. Most plants are grown from pre-germinated seeds and a known weight of the inoculum is applied to experimental host plant in pots. These pots are maintained for 14 days after which they are harvested, the root length measured and then stained. The resulting entry points are counted to ascertain the infectivity potential.

## **6.2 Root Clearing and Staining**

Before AM colonization can be observed, root samples need to be taken through a series of stages during which roots are **cleared** in potassium hydroxide, **bleached** in alkaline hydrogen peroxide, acidified in hydrochloric acid and stained.

The preparation of staining reagents is done under a fume hood cupboard. Strict measures are observed in the preparation process and caution taken in the handling and disposal of reagents.

The process involves sampling, clearing, staining and analysis of roots to see AM structures

### **B.6.3 Materials and Equipment**

Water bath: heat to 90°C/ Oven/Autoclave

Test tubes and holders

Tweezers

Slides

Cover slips

Dissecting or low magnification microscope

Compound microscopes

### **B.6.4 Preparation of Solutions**

2.5% KOH (25g KOH in 1000 ml water)

10% HCL

Lactic acid solution -

875 ml lactic acid

63 ml glycerin

63 ml distilled water

The reagents used in the preparation of the staining solution are KOH (caustic), alkaline hydrogen peroxide (irritant), hydrochloric acid (toxic), glycerol and trypan blue. This comprises of preparation of staining reagents, root staining, slide preparation and assessment of AMF colonization.

In the process of staining many samples, it is recommended that perforated plastic tubes be used. The tubes are placed on racks and the racks immersed into the solutions.

### B.6.5 Staining Reagents

- 1) Trypan Blue: 0.05% trypan blue (500 ml glycerol, 450 ml water, 50 ml of 1% HCL and 0.5g trypan blue) staining reagent was added and placed in the oven for 1 hour at 70 °C.
- 2) Add 0.1 g. acid fuchsin to 1 litre lactic acid solution (0.01% acid fuchsin-lactic acid solution); Heat at 90°C. for 10 to 60 minutes.
- 3) Chlorazol Black E (0.03 % w/v) stain in lactoglycerol solution (1:1:1 lactic acid:glycerol:water) (Brundrett *et al.* 1983).
- 4) Ink (black = Shaeffer, blue = Pelikan)

*One could use either of them. Not all of them are required at the same time*

### B.6.6 Assessment of arbuscular mycorrhizal infection

#### B.6.6.1 Gridline intersect method

After staining, AM structures can be observed in the cortical cells of the roots using a low power microscope (**dissecting (stereo-) microscope**).

$$\text{Infection (\%)} = \frac{\text{Total infected}}{\text{Total uninfected}} \times 100$$

Under the **dissecting microscope**, intracellular structures such as arbuscules and coiled hyphae and hyphae are difficult to differentiate; only intraradical spores and vesicles are easily identified.

#### B.6.6.2 Compound microscope technique

AM structures (**appressoria, intercellular hyphae, intracellular coiled hyphae, arbuscules and vesicles**) can be observed in the root cells using a compound microscope. This method enables different AM structures within the root to be quantified and related to function.

### B.6.7 Contamination assessment

The AMF product shall be assessed to determine whether contaminant could be detected at a dilution level of  $10^{-5}$ . The general dilution techniques in microbiology techniques apply.

### B.6.8 pH

- a) Prepare a suspension of 25 g mycorrhiza in 50 ml distilled water
- b) Shake on a rotary shaker for 2 hours
- c) Filter through Whatman No 1 or equivalent filter paper
- d) Determine the pH of the filtrate

### **B.6.9 Moisture content**

Applicable to solid carrier based products only.

- a) Weigh 5 g of finished product in appropriate, pre-weighted, clean, and dry container
- b) Heat in oven at 65°C until constant weight is attained (approximately 5 hours)
- c) Cool in a desiccator and take weight using a precision balance
- d) Moisture content is reported from % loss in weight according to the following calculation

$$\text{Moisture by weight (\%)} = \frac{B-C}{B-A} \times 100;$$

where, A is the weight of the container itself, B is the weight of the container plus sample before drying, and C is the weight of the container and the sample after drying.

### **B.6.10 Particle size**

Applicable to powder formulation only.

- a) Weigh 25 g of finished product in appropriate, clean, and dry container
- b) Transfer the weighed product in a 250 micron International Standard (IS) sieve
- c) Proceed with sieving until there is no more product passing through the 250 micron IS sieve
- d) Weigh the residual product retained by the 250 micron IS sieve after sieving
- e) The finished product passing through the 250 micron IS sieve is determined according to the following calculation:

$$\text{Finished product passing through the 250 Micron IS sieve (\%)} = \frac{A-B}{A} \times 100;$$

where A is the weight of finished product transferred in the 250 micron IS sieve and before sieving and B is the weight of the residual product retained by the 250 micron IS sieve after sieving.

## **B.7 Conclusion**

Arbuscular mycorrhizae fungi products intended to be sold in market place shall be evaluated for quality control to determine the amount of viable propagules, infectivity potential, level of contamination, and pH value. Depending on the carrier material and the nature of the product moisture content and particle size should be determined. Any AMF product found in the marketplace that has not undergone the adequate quality control as stipulated here for whatsoever reason should be subject to enforcement action. Only AMF products that meet the quality control criteria should be granted a registration certificate for selling and use.

## **8 References**

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### **Annex 3: Standard operating procedure for testing the quality of trichoderma inoculants**

*Note: The standard operating procedure retained by the Kenya Bureau of Standards (KEBS) has been used in this manual as an example. However, similar SOPs have been developed in Ethiopia, Ghana, Nigeria, Tanzania, and Uganda. For training or application, the country-specific SOP will have to be used. However, for countries without their own SOP, they can customize this one. The list of reference includes the various materials used to compile the SOP.*

#### **INTRODUCTION**

*Trichoderma spp* are filamentous fungi widely distributed in the soil, plant material, decaying vegetation, and wood. They are facultative anaerobes growing saprophytically or as a parasite on other fungi. Different species of this fungus are used in agriculture against various phytopathogens of crops in outdoor planting and green house. Such species as *T. harzianum*, *T. hamatum*, *T. lignorum*, *T. viride*, *T. koningii* and their biotypes have the most biological and commercial importance. The active components of biopesticides made on the base of this fungus-antagonist are their spores, mycelia and products of metabolism.

#### **DEFINITIONS**

**Biofertilizer** is a term widely used term meaning “microbial inoculant” usually refer to preparations of microorganisms that may be a partial or complete substitute for chemical fertilization (like rhizobial inoculants).

**Biopesticides:** Living organisms or natural products derived from these organisms which suppress pathogen populations such as microbial pesticides.

**Bioinoculants:** Living organisms containing specific strains of specific bacteria, fungi and algae which; fix atmospheric nitrogen, make nutrients soluble and available, collect and store nutrients, provide physical barriers to pests and pathogens, stimulate plant growth, and decompose organic residue.

**Bio-enhancers:** Substances that increase the bioavailability of active ingredients, vitamins and nutrients

**Fungi:** Multicellular organism, separate Kingdom from plants, animals, protists and bacteria.

**Trichoderma:**A saprophyte fungus, which belongs to the family Hypocreaceae, the division Ascomycota.

**Contamination:** Any other microorganism other than the active agent not declared on the label.

**Aseptic Conditions/Aseptically:** Environment where no microorganisms is present. It can be obtained by using a Bunsen burner to create an aseptic zone which is the aspheric area around the flame with a diameter of approximately 15 cm or a Laminar flow hood with sterile air continuously produced and present in the hood. The high pressure of air flow from the hood prevents the air from outside to come inside and contaminate the environment in the hood; aseptic conditions are also created by cleaning surface with 70% ethanol.

**Contaminated by microorganisms:** Every plate, tube, pipette, or other instruments (glassware, pestles, eppendorff tube...) which has been in contact with microorganisms and cannot be sterilized by the flame of a Bunsen burner or any other contamination procedure is considered as contaminated.

**Contaminated by toxic chemicals:** Every tube, flasks, pipette or other instruments which has been in contact with toxic chemicals is considered contaminated

**Good Laboratory Practices (GLP):** The principle of GLP have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a management concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored,, recorded and reported. Its principle also include the protection of man and the environment.

**Mother tube/plate/products:** Tube/plate/product which the bacteria are picked from. The result of the growth of this inoculation is considered as the daughter which can become the mother for the next inoculation.

**Substrate:** Any material, that serves as source of energy for an organism.

**Carrier:** Delivery media of live microorganisms from the production to the field. The carrier is the major portion of inoculant. There is presently no universal carrier or formulation available for release of microorganism into soil. Materials and types of formulations of carriers vary: Slurry, powder, peat, liquid. A good carrier should have the capacity to deliver the right number of viable cells in good physiological condition at the right time.

**Growth Medium:** growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms

**Agar:** Agar consists of a mixture of agarose and agaropectin. Agar is used to provide a solid surface containing medium for the growth of bacteria and fungi.

**Aseptic :** an environment or procedure that is free of contamination by pathogens

## **Abbreviations**

**°C:** degrees Celsius

**mg:** milligram

**g:** gram

## **1.2 PURPOSE**

The Standard Operation Procedure (SOP) is intended to guide laboratories appointed by regulatory agencies in screening of *Trichoderma* commercial products for quality. Based on the information on the product label, the screening is to confirm the strains which influence environmental parameters that are of great importance when planning of strains for biocontrol. This is to guarantee customers quality product devoid of contaminants. This exercise is also to verify if there are any contaminants that can reduce the efficacy of the product or are harmful to plants, human, animals and the environment. The SOP outlines the procedures provided by the regulatory agency to generate quality data as stipulated on the label and of acceptable standards set by regulatory agencies.

## **1.3 SCOPE:**

This procedure describes the process of sample preparation and screening of *Trichoderma* products for quality and reporting of results of analysis.

## **1.4 RESPONSIBILITIES**

The technologist will be responsible for carrying out *Trichoderma* quality tests. The results will be verified by the head of section, a report done and sent to the KEPHIS and copied to the clients.

## **2.0 MATERIALS AND REAGENTS**

### **2.1 Media**

*Trichoderma* Selective Media (TSM)

Oat Flour Agar (OFA)

Czapek Dox Agar (CDA)

Rose Bengal Agar (RBA)

Potato Dextrose Agar (PDA)

Malt Extract Agar (MEA)

### **2.2 Materials**

Petri dishes (you can use disposable type)

Media preparation bottles

Weighing boats



## 2.3 Instruments /Equipments

Safety cabinet

Autoclave

Incubator

Bunsen burner

Weighing balance

Calibrated Compound microscope

Dissecting microscope

Pestle and mortar

Isolating needle

Spatula

## 2.4. Sterilizing and Preparation Procedure for Plates

If plates are glass, sterilize the sampling and plating equipment with dry heat in a hot air oven at not less than 160 °C for not less than 2 hours.

Sterilize the media by autoclaving at 121° C for 20 minutes. To permit passage of steam into and from closed container when autoclaved, keep stoppers slightly loosened or plugged with cotton. Air from within the chamber of the sterilizer should be ejected allowing steam pressure to rise.

## PROCEDURE

### 2.5 Isolation of *Trichoderma* from commercial products and other substrates

#### 2.5.1 Isolation Techniques

##### (i) Warcup's Soil Plate Technique

Take 5mg of product and distribute evenly over the surface of the solidified media (Warcup, 1950). You can grind the product (if in granular formulation) sample in a mortar with a pestle and pass the product through a 1.7mm sieve if the product is not fine. This helps in separation and recovery of colonies. The isolation media can be any of the following: Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Oat Flour Agar (OA), Rose Bengal Agar (RBA), Czapek Dox Agar (CDA), or *Trichoderma* Selective Media (TSM) (Appendix 1). The last is selective for other fungi and bacteria.

Incubate the plates for 5 days at 28°C.

Transfer all colonies determined to be *Trichoderma* according to Watts *et al.*, (1988) and Rifai (1969), onto fresh media for purification. Incubate the plates for 5 days at 28°C.

From the bacteria free cultures you now have, prepare single spore cultures for use in identification (Appendix 2).

Colonies are usually first white then develop yellowish tints until they become various deep shades of green.

Conidiophores will arise as branches of aerial mycelia, septate, and grow up to 70 cm in height.

If you used TSM, transfer *Trichoderma* colonies to PDA. Incubate the plates for 5 days at 28°C.

Observe characteristics of the colony such as colour of colony, pigmentation diffusing into growth medium, liquid droplets. The identification keys listed below will guide you on the characters to look for.

Prepare slides for further examination and measurements of features.

Use recommended identification keys by Watts *et al.*, (1988) and Rifai (1969). The website below also provides an interactive key by Samuels GJ:

[http://nt.ars-grin.gov/taxadescriptions/keys/Trichoderma\\_Index.cfm](http://nt.ars-grin.gov/taxadescriptions/keys/Trichoderma_Index.cfm)

This link will lead you to a morphological key with descriptions and over 500 images for the 32 species of *Trichoderma* compiled by Dr. Gary J. Samuels from USDA, Beltsville, USA.

The key also includes species of *Hypocrea* that have named *Trichoderma* stages.

In cases where the key requires growth rates for identification, measure radial growth daily for 14 days.

*Trichoderma* grows fast and sporulates heavily. This requires very careful preparation of slides for meaningful examination. Pick very thin growth for your slide preparation (Appendix 3).

Conidiophore branching is an important identification characteristics. In case you are unable to prepare good slides from your Petri dishes, prepare slide cultures for this (Appendix 4)

It is well known that due to homoplasy of morphological characters it is often impossible to discriminate species. For this reason it is advisable to do molecular identification using the following phylogenetic markers (a) ITS 1 and ITS 2 (internal transcribed spacer 1 and 2 of rRNA gene cluster) (b) Tef 1 (translation elongation factor 1-alpha encoding gene)

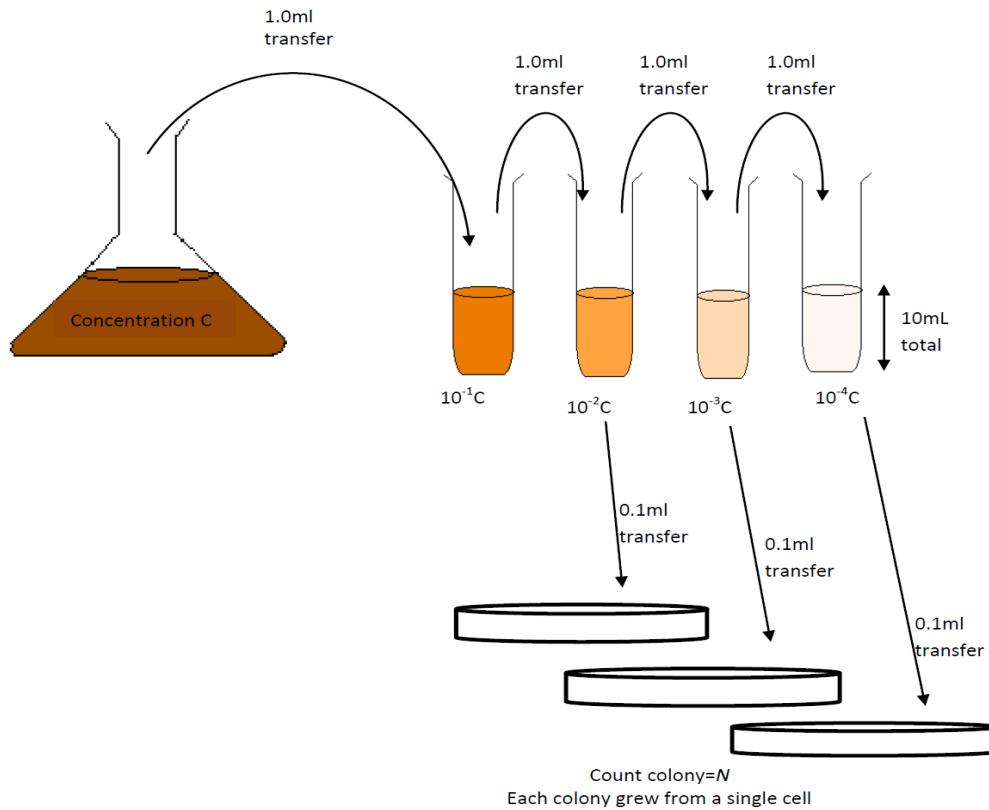
## **(ii) Product Dilution Plate Technique**

The dilution media is 0.05% Water AgarWA.(see Annexe 2)

Grind the product sample in a mortar with a pestle.

Dissolve 1g by agitation in 9 ml of sterile 0.05 % WA while liquid is viscous enough to be evenly distributed.

Subject the suspension to a ten-fold dilution series in sterile 0.05 % WA up to 10<sup>-4</sup> dilution, Figure 1.



### 2.5.2: Serial dilution

Transfer 1ml of the product suspension from the  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dilutions to a 100-mm diameter Petri dish using a pipette and spread evenly across the agar surface. Use a glass ‘hockey stick’ applicator to spread the suspension.

Use the various isolation media described above.

Petri dishes containing agar to be used with product dilutions should be allowed to dry for 3-5 days before use. The drier media more quickly soaks up excess water in the suspension, which helps minimize bacterial contamination.

Follow procedure outlined in section (i) No. 2-8 above

## APPENDIX 1

### MEDIA FOR ISOLATION AND GROWTH

#### Potato Dextrose Agar (PDA)

The medium contains:

20 g dextrose

20 g PDA

1 L distilled water

Add all these to a media preparation bottle and autoclave at 15 lbs pressure (121°C) for 15 minutes. Let to cool until you can hold by hand. Dispense 10-20ml of the sterile molten agar into Petri dishes aseptically.

#### Rose Bengal Medium (RBM)

Mycological Peptone	5.0g
Glucose	10.0g
Di-Potassium hydrogen phosphate	1.0g
Magnesium sulfate	0.5g
Rose Bengal	0.05g
Chloramphenicol	0.1g
Bacteriological agar	15.5g

Dissolve ingredients in water and autoclave for 15 min at 121°C.

#### Czapek Dox Agar (CDA)

Sucrose	30g
Sodium nitrate	2g
Dipotassium phosphate	1g
Magnesium sulphate	0.5g
Potassium chloride	0.5g
Ferrous sulphate	0.01g
Agar	15g
Distilled water	1L

Final pH (at 25°C) should be 7.3±0.2

If using commercial product, suspend 49.01 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates

### Oatmeal

Oatmeal or oat flour	60		
Agar	12.5		g
Distilled water	1L		

### Agar (OA)

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH is 6.0 and requires no adjustment

### Malt Extract Agar (MEA)

Malt extract	30g
Mycological peptone	5g
Agar	15g
Distilled water	1L

Suspend 50g in 1 litre of distilled water and boil to dissolve. Sterilise by autoclaving at 15 lbs pressure (121°C) for 15 minutes

### *Trichoderma* Selective Medium (TSM) (Elad *et al.*, 1981)

#### Basal medium

MgSO <sub>4</sub> (7H <sub>2</sub> O)	0.2 g	
K <sub>2</sub> HPO <sub>4</sub>	0.9 g	
KCl	0.15 g	
N H <sub>4</sub> NO <sub>3</sub>	10	g
D glucose anhydrous	3g	
Agar	20 g	
Distilled water	950ml	

Autoclave at 121 C for 15 min.

The	biocidal		ingredients
Chloramphenicol (crystallized)		0.25	g
p-dimethylaminobenzenediazo	sodium	sulfonate0.3	g
Pentachloronitrobenzene		0.2g	
Rose bengal		0.15	g

Mix in 50 ml of sterilized (autoclaved at 121 C for 15 min) distilled water and add to the autoclaved basal medium.

**Water Agar**

20 g Agar

1 liter distilled water

## APPENDIX 2

### SINGLE-SPORE TECHNIQUE

#### EQUIPMENT REQUIRED

Dissecting microscope (30 – 40X) total magnification; with light source below the observation stage) or a micro-manipulator.

Sharp needle; or flattened, arrow-shaped needle

2-3 test tubes (per culture) filled with 10-15 ml sterile, distilled water

2-3 Difco Bacto agar plates (per culture), thin-medium thickness agar, 90 mm diameter.

4 Difco plates (per culture), thinly poured agar, 90 mm diam, no additives or antibiotics.

5 ml syringe, sterile, disposable.

#### GENERAL METHOD

**NB!!** Single-sporing should only be done from *Trichoderma* cultures which are free of bacterial and/or mite contamination. Contaminated cultures should first be cleaned up, e.g. plating onto PDA ring plates to remove bacteria. Single-sporing is useful to separate 'mixed' cultures and is absolutely essential for *Trichoderma* strains which are to be deposited in any Culture Collection or used for identification, mycotoxin, genetic or molecular studies.

#### Procedure

Under aseptic conditions, remove a needle-point of spore mass and place in the first of three tubes of water filled with 10-15 mls sterile distilled water. Shake for approx. 2 – 4 sec.

From this first tube, transfer 1 ml suspension to the second tube with a sterile syringe and shake. Make a similar dilution from the second tube into the third tube. Pour most of the content of each tube onto the surface of a thin Bacto agar plate. Swirl plate gently and pour off the excess suspension.

Keeping these Bacto agar plates inverted, incubate them at a slight angle, at 20 – 25°C for 16 – 24 hours. The angle allows water to drain away from the spores attached to the agar surface.

Examine the plates under the dissecting microscope and select the dilution with the best spread of germinated spores. The best magnification to work is at 30-35 X. Adjust the light source so that the germinated spores are clearly visible.

The exact plane of view of the spores can be established by touching the agar surface with a needle, then focus on this hole and find the spores lying nearby.

Germinated spores should lie well clear of each other, with no overlapping of germ tubes or developing hyphae!

Select each 'germling' and while viewing under the microscope, make four incisions to obtain a  $\pm 1 \text{ mm}^2$  block.

Lift out the agar block, making sure that the conidium is not dislodged from the agar in the process. Place each block in the centre of a PDA plate. Make two plates per culture/strain. Incubate the plates, preferably inverted, at 25°C.

## APPENDIX 3

### MAKING A BASIC MICROSCOPE PREPARATION

#### Observing *Trichoderma*

##### Procedure

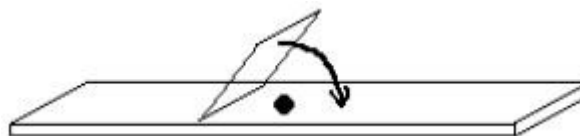
Place a small drop of stain or mountant in the middle of the slide. If the material is dark, use colorless lactophenol; if it is colourless use a stain e.g lactophenol cotton blue.

Using a fine needle pick off a small portion of the mycelium of the fungus from the substrate or Petri plate provided and place on the mountant. Tease it out with as little disturbance as possible.

Take a clean cover slip and hold it on one side, then let it slip onto the liquid from one side without trapping air bubbles underneath, Fig 2.

Press the cover slip down gently. There must be just enough mountant or stain to suspend the cover slip.

Observe using a compound microscope.



#### Preparation of a slide for microscopic examination

## APPENDIX 4

### SLIDE CULTURE TECHNIQUE FOR FUNGI

#### Materials Required



**Culture:**

7-10 day old fungal culture

**Media:**

PDA

**Equipments:**

Sterile Petri dish

Filter paper (9cm diameter)

U-shaped glass rod

Microscope slides and coverslips (Sterile)

PDA plate with mixed culture of fungi

Sterile PDA plate

Lactophenol cotton blue stain

Glass capillary tube

Scalpel

Inoculating needle

Sterile distilled water

95% ethanol

Forceps

**Procedure:****Slide Culture Preparation**

Aseptically, with a pair of forceps, place a sheet of sterile filter paper in a Petri dish.

Place a sterile U-shaped glass rod on the filter paper. (Rod can be sterilized by flaming, if held by forceps.)

Pour enough sterile water (about 4 ml) on filter paper to completely moisten it.

With forceps, place a sterile slide on the U-shaped rod

Gently flame a scalpel to sterilize, and cut a 5 mm square block of the medium from the plate of PDA (Fig 3).

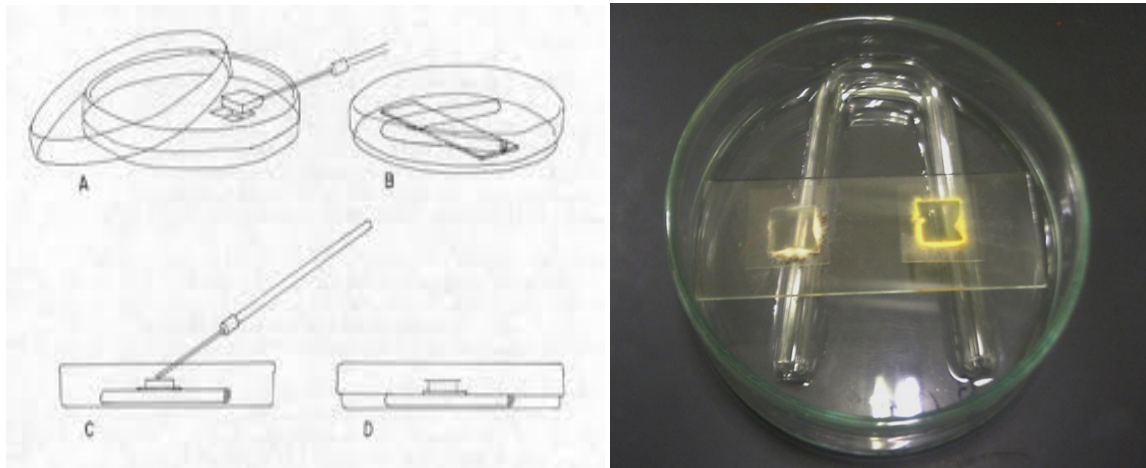
Pick up the block of agar by inserting the scalpel and carefully transfer this block aseptically to the centre of the slide.

Inoculate four sides of the agar square with spores or mycelial fragments of the fungus to be examined. Be sure to flame and cool the loop prior to picking up spores.

Aseptically, place a sterile cover glass on the upper surface of the agar cube.

Cover the Petri dish and incubate at room temperature for 48 hours.

After 48 hours, examine the slide under low power. If growth has occurred there will be growth of hyphae and production of spores. If growth is inadequate and spores are not evident, allow the mold to grow for another 24–48 hours before making the stained slides.



**Fig 3: Preparation of slide culture**

### **B) Application of Stain**

Place a drop of lactophenol cotton blue stain on a clean microscope slide.

Remove the cover glass from the slide culture and discard the block of agar.

Add a drop of 95% ethanol to the hyphae on the cover glass. As soon as most of the alcohol has evaporated place the cover glass, mold side down, on the drop of lactophenol cotton blue stain on the slide. Examine the slide under microscope

### **Advantages of slide culture:**

It is a rapid method of preparing fungal colonies for examination and identification.

Permits fungi to be studied virtually in situ with as little disturbance as possible

Fungi are identified mostly by close examination of its morphology and the characteristics it possess. In slide cultures, we are growing the fungi directly on the slide on a thin film of agar. By doing this, there is no need to remove a portion of the fungus from a culture plate and transfer it to the slide. So there is less chance for the features that are key to identification, notably the spore-bearing structures, to be damaged.

**NB**

*The laboratory technologists should be competent, well conversant with good laboratory practice ( GLP) and safety regulations.*

*The laboratory should be well designed and equipped with equipment to run the test.*

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Watts R, Dahiya J, Chaudhary K, et al. Isolation and Characterization of a New Antifungal Metabolite of *Trichoderma reesei*. *Plant and Soil* 107: 81-84. 1988.

### Specific Quality requirements of Trichoderma

	<b>Parameter</b>	<b>Requirements</b>
	Base	Carrier based (dry powder, pellets or granules) Liquid based
	Viable cell count (CFU)	Minimum $2 \times 10^6$ /ml or g
	Contamination level	No contamination at 104 dilution Zero contamination for pathogens
	pH	6.0 -7.5
	Particle size (carrier based)	All material shall pass through 0.15- 0.212 mm IS sieve
	Moisture % by weight for carrier based carrier	30-40%
	Shelf life	Should have at least 6 months 12 months for at 20 degrees centigradeoC oC for vacuum packs
	stability	30 degrees centigradeoC and 65% Relative humidity
	Infectivity potential	90% of inoculated plant roots tested positive for Trichoderma

## **Annex 4: Standard operating procedure for testing the quality of azospirillum inoculants**

*Note: The standard operating procedure retained by the Plant Protection Regulatory Services Directorate (PPRSD) of the Ministry of Food and agriculture of Ghana has been used in this manual as an example. However, similar SOPs have been developed in other COMPRO-II countries. For training or application, the country-specific SOP will have to be used. However, for countries without their own SOP, they can customize this one. The list of reference includes the various materials used to compile the SOP.*

### **Description of selected terminologies used in this SOP**

**Biofertilizer:** a substance which contains living microorganisms which colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrient and/or growth stimulus to the target crop, when applied to seed, plant surfaces, or soil.

**Bioenhancers:** Substances that increase the bioavailability of active ingredients, vitamins and plant nutrients.

**Biostimulants:** Dormant strains of positive product containing strains of specific bacteria, fungi or algae, which take up nutrients and make them available to plants; collect and store available nutrients; enhance uptake of nutrients; provide physical barriers against pathogens; stimulate plant growth; and or enhance decomposition of organic residues.

**Bacteria:** A Kingdom of single celled microorganisms whose cells lack membrane-bound nucleus (prokaryotic) that live in soil, water, [acidic hot springs](#), [radioactive waste](#), and the deep portions of [Earth's crust](#). Bacteria also live in [symbiotic](#) and [parasitic](#) relationships with plants and animals.

**Symbiotic bacteria:** Species of bacteria living in a mutual relationship with plants where they convert atmospheric nitrogen to nitrates which the plants utilizes for its development. They in turn obtain sugars from the plants in the association.

**Azospirillum;** A free - living nitrogen -fixing bacterium closely associated with grasses. It also produces phytohormones.

**Contamination:** presence of other microorganism other than rhizobia not declared on the label.

**Aseptic Conditions /Aseptically:** Environment where no microorganisms is present. It can be obtained by using a Bunsen burner to create an aseptic zone around the flame with a diameter of approximately 15 cm or a Laminar Flow Hood with sterile air continuously produced, and present in the hood. Aseptic conditions are also created by cleaning surfaces with 70% ethanol.

**Contaminated by microorganisms:** Every plate, tube, pipette, or other instruments (glassware, pestles, eppendorff tube, etc) which has been in contact with microorganisms and cannot be sterilized by the flame of a Bunsen burner is considered as contaminated by microorganisms.

**Contaminated by toxic chemicals:** Every tube, flasks, pipette or other instruments that has been in contact with toxic chemicals are considered contaminated by toxic chemicals.

**Good Laboratory Practices (GLP):** The principles of GLP have been developed to promote the quality and validity of results of the analysis conducted in a laboratory. It is a management concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principle also includes the protection of man and the environment.

**Mother tube / plate / products:** Tube / plate / product which the bacteria are picked from. The result of the growth of this inoculation is considered as the daughter, which can become the mother for the next inoculation.

**Substrate:** Any materials that serves as source of energy for an organism.

**Carrier:** A delivery vehicle of live microorganisms from the factory to the field. The carrier makes the largest proportion of inoculant. Materials and types of formulations of carriers varies; could be in form of slurry, powder, peat or liquid.

**Quality control:** A process seeking to ensure that the *quality* of Azospirillum products based on specific specifications is maintained or improved and manufacturing errors reduced or eliminated

## 1.0 Introduction

Inoculation of plants with azospirillum has the overall objective of delivering to the plant's rhizosphere the maximum number of appropriate azospirillum to ensure better root architecture and elongation. Azospirillum has the capacity to produce phytohormones to promote plant growth. The delivery of high quality azospirillum inoculants to farmers is one of the cardinal objectives of the PPRSD. The quality and efficacy of azospirillum inoculants

is determined by the strain (s), the abundance of cells as well as the formulation. The desired characteristics of inoculants are therefore defined by high strain density, significantly long shelf life, and absence of, or minimum contamination. Thus, the products must meet or exceed regulatory quality requirements.

This document is intended to provide procedures for carrying out requisite tests for inoculant quality in Ghana.



## 2. Purpose

The Standard Operation Procedure (SOP) is intended to guide laboratories appointed by PFRD-PPRSD in screening of Azospirillum products for quality. Based on the information on the product label, the screening is to confirm the strains, populations and ability to produce effective nodules. This is to guarantee customers quality product devoid of contaminants that can reduce the efficacy of the product or are harmful to plants, human, animals and the environment. The final outcome is to guide the regulatory agency on the quality of the product and its suitability for use by farmers. The SOP outlines the procedures provided by the PFRD-PPRSD to generate quality data as stipulated on the label and of acceptable standards.

## 3. Minimum criteria for inoculant quality

In addition to the requirements in the registration guidelines related to labeling, efficacy, and safety, azospirillum inoculants intended for sale in Ghana shall meet the quality criteria listed below. The PFRD-PPRSD reserves the right to request additional information related to the quality of the product when deemed necessary.

Minimum viable cell:  $10^7$  cells /g of viable rhizobia.

Contamination level: no contaminant at  $10^4$  dilutions

pH: 7.0 – 8.0

Moisture content (per cent by weight of finished product): maximum of 35 – 40 % (in case of solid carrier based)

Particle size: 90 % of the material shall pass through 0.15 - 0.212 mm IS sieve.

## 4.0 Method of Analysis for Azospirillum

### 4.1 The apparatus required:

- Graduated pipettes – 1 and 10 ml
- Dilution bottles or flasks
- Petri dishes – uniform, flat-bottomed
- Hot-air oven
- Autoclave
- Incubator

- Hand tally or mechanical counting device
- pH meter.

## **4.2 Reagents**

### **4.2.1 Medium**

Use N-free semisolid medium (Nfb) of the following composition for preparation of MPN tubes.

DL-Malic acid	5.0
K <sub>2</sub> HPO <sub>4</sub>	0.5
MgSO <sub>4</sub> 7H <sub>2</sub> O	0.2
NaCl	0.1
CaCl <sub>2</sub>	0.c 02
Trace element Soln.	2.0 ml
Fe EDTA (1.64% Soln.)	4.0 ml
Vitamin soln.	1.0 ml
KOH	4.0 ml
Bromothymol blue (0.5% aq.)	2.0 ml
Adjust pH	6.8-7.0 with KOH
For semi solid add agar	1.75 g
For solid medium add agar	15.0 g

#### **3.2.1.1 Trace element solution (g/litre)**

Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.2
MnSO <sub>4</sub> H <sub>2</sub> O	0.235
H <sub>3</sub> BO <sub>3</sub>	0.28
CuSO <sub>4</sub> 5H <sub>2</sub> O	0.008
ZnSO <sub>4</sub> 7H <sub>2</sub> O	0.024
Distilled water	1000 ml

Use 2 ml of this solution in one litre of Nfb media.

## **4.3 Sterilization and preparation of MPN tubes**

3.3.1 Prepare Nitrogen free Bromothymol Blue malate medium. Boil to dissolve agar. Quickly dispense 10 ml molten media in 15 x 150 ml test tubes or screw capped culture tubes and close either with cotton plugs or screw caps. Minimum of 25 such tubes shall be needed for each sample. Sterilize the tubes by autoclaving at 121 °C for 20 minutes, as in Rhizobium.

#### 4.4 Preparation of serial dilution for MPN count

Dispense 30 g of Azospirillum biofertilizers in 270 ml of sterile water and shake for 10 minutes on a reciprocal shaker. Make serial dilutions up to 10<sup>-8</sup> dilution. Pipette 1 ml aliquots of 10<sup>-4</sup> to 10<sup>-8</sup> dilution and deliver it to screw cap tubes or test tubes containing N-free semi solid Nfb media.

#### 4.5 Incubation of tubes

Label the tubes and incubate at 36 + 10 °C for 3 - 4 days in vertical position in a test tubes stand. Do not disturb the medium during the entire period of incubation.

P1	P2	Most probable number for indicated values of P 3					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.16	0.17
1	3	0.089	0.10	0.13	0.16	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24

2	0	0.046	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16.0	--

#### **4.6 Counting**

1. Count the tubes which have turned blue and have developed typical white subsurface pellicle.

Count the tubes as + ve or – ve for the presence of sub-surface pellicle and consider for the purpose of calculation.

#### **4.7 Method for Estimating MPN Count**

1. To calculate the most probable number of organisms in the original sample, select as P1 the number of positive tubes in the least concentrated dilution in which all tubes are positive or in

which the greatest number of tubes is + ve, and let P2 and P3 represent the numbers of positive tubes in the next two higher dilutions.

2. Then find the row of numbers in Table 1 in which P1 and P2 correspond to the values observed experimentally. Follow that row of numbers across the table to the column headed by the observed value of P.

3. The figure at the point of intersection is the most probable number of organisms in the quantity of original sample represented in the inoculum added in the second dilution. Multiply this figure by the appropriate dilution factor to obtain the MPN value.

$$\text{Azospirillum count per gram of carrier} = \frac{\text{Value from MPN table} \times \text{Dilution level}}{\text{Dry mass of product}}$$

#### **4.8 Maintenance and preparation of pure culture and quality control at Broth stage**

1. Maintain pure culture of Azospirillum on nitrogen free bromothymol blue medium and maintain as solid medium.

2. Transfer a loopful of pure culture to each of the agar culture tube aseptically in an inoculation room and incubate  $37 \pm 2$  °C for three days and keep in undisturbed. Always keep pure culture below 5 °C.

#### **4.9 Preparation of Inoculums culture and Mass culture:**

Inoculums culture and mass culture of this standard shall be prepared as described for Rhizobium of this standard.

#### **5.0 Quality Control Test Recommended at Broth Stage**

##### **Quality Test**

Check for free from contaminants by preparing slide and observing under microscope.

The pH of bacterial broth shall normally be between 7.0 to 8.0.

Gram staining test shall be carried out as described for Rhizobium of this standard.

See the colour change in the media after 24 hours from inoculation. The colour will change from green to blue.

Watch the pellicle just below the surface of the media. It is checked on the third day after keeping inoculated broth undisturbed.

#### **5.1 Quantitative Test**

Most probable Number (MPN). The counts of Azospirillum in the final broth from shake culture or fermenter shall be not less than 10<sup>8</sup> to 10<sup>9</sup> cells / ml. Otherwise the broth should be rejected.

#### References

**Bio-fertilizers and Organic Fertilizers in Fertilizer (Control) Order (1985).** National Centre of Organic Farming Department of Agriculture and Cooperation, Ministry of Agriculture, Govt of India, CGO-II, Kamla Nehru Nagar Ghaziabad, 201 001, Uttar Pradesh

**Bhattacharyya, P. and Tandom (2012).** Biofertilizer handbook: Research – Production – Application. Fertilizer Development and Consultation Organization 204-204A Bhanot Corner, Pamposh Enclave, New Dehli 110048 (India). pp 190. ISBN: 81-85116-64-4.

## **Annex 5: Standard operating procedure for testing the quality of azotobacter inoculants**

*Note: The standard operating procedure retained by the Ethiopian Standards Agency has been used in this manual as an example. However, similar SOPs have been developed in other COMPRO-II countries. For training or application, the country-specific SOP will have to be used. However, for countries without their own SOP, they can customize this one. The list of reference includes the various materials used to compile the SOP.*

**ETHIOPIAN  
STANDARD**

**ES 3907-4: 2015**

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**Fertilizers-Biofertilizers-part 4:-Azotobacter  
specification and test method.**

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ICS: 65.080

Published by Ethiopian Standards Agency  
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## **Foreword**

This Ethiopian Standard has been prepared under the direction of Technical Committee for Fertilizer (TC 15) and published by the Ethiopian Standards Agency (ESA).

The draft document (Working Draft, WD) has been submitted to the Secretariat by the Ethiopian Institute of Agricultural Research/EIAR/.

### **References**

During the preparation of this standard reference was made to the following documents:

*EAS 456: 2007- East African Organic Products Standard*

*The FNCA biofertilizer manual 2006*

*Indian bio fertilizer standards-*

*IS 8268:2001-Standard for Rhizobium*

*IS 9138:2002-standard for Azotobacter*

*IS 14806:2000-Standard for Azospirillum*

*IS 14807:2000-Standard for phosphate solubilizers*

Acknowledgement has been made to the said organization for their effort of presenting the draft document and for the assistance derived from the above sources.

## Introduction

Fertilizers directly increase soil fertility by adding nutrients. Biofertilizers add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing Phosphate or nutrient mobilization to stimulate plant growth through the synthesis of growth promoting substances. They can be grouped in different ways based on their nature and function. Most biofertilizers are produced from microorganisms such as *Rhizobium*, *Azotobacter*, *Azospirillum*, Phosphate solubilising bacteria. Other types include mycorrhizal biofertilizers, potassium mobilizing biofertilizers and zinc solubilising biofertilizers.

The use of biofertilizers offer economic and ecological benefits by way of soil health improvement and fertility. This standard is in line with the fertilizer policy of Ethiopia which aims to ensure availability of high quality biofertilizer products for efficient use by the farmers

Regulations on inoculant quality vary from country to country and no set of international standards exists. Brazil, Canada, France, and Uruguay have regulatory authorities supported by legislation. Australia, South Africa, and New Zealand have quality control programs in which inoculant manufacturers participate voluntarily. In many other countries such as USA and UK product quality standards are left to the discretion of the manufacturers. Whether legislatively set or internally established by the manufacturer, standards for inoculants products should be a compromise between theoretical possibilities and practical limitations. However, without defined standards quality control cannot work (Thompson, 1991a). There exists a consensus that the establishment of standards, whether voluntary or imposed, has improved legume inoculant quality.

The objective of this standard is to ensure that biofertilizers on the market are appropriately tested through the quality criteria provided while ensuring that farmers obtain only certified products and as well aid the industry in the manufacture of quality biofertilizers. This standard will also promote the safe use of biofertilizers and promote fair trade.

## Fertilizers-Biofertilizer Azotobacter Specification and test method

### 1. Scope

This standard prescribes the requirements, method of sampling and tests for Azotobacter biofertilizer.

### 2. Normative reference

The following referenced documents are indispensable for the application of this Ethiopian standard. Only the edition of the documents (including any amendments) shall be applicable.

**ES 3907-1:**-Fertilizers- Biofertilizer — part -1: *Rhizobium* Specification and test method

**ES ISO 14001:** Environmental management systems - requirements with guidance for use

### 2. Terms and Definitions

For the purpose of this standard the definition in ES 3907-1 shall apply: .

### 3.0. Requirements

#### 3.1 General requirements

##### 3.1. Biofertilizers shall:

- 3.1.1. contain competent, persistent and effective strain in minimum recommended population.
- 3.1.2. contain no more than the maximum allowed level of contamination.
- 3.1.3. have single or a combination of effective strains.
- 3.1.4. contain no pathogenic organisms that affect the biophysical environment.
- 3.1.5. contain carrier materials that are not harmful to the environment.
- 3.1.6. have at least one of the following effects:
- 3.1.7. should also have characteristics of mineral solubilisation/mobilization,ability to stimulate plant growth or Other desirable functions to inhance tolerance to pest damage and amelioration of the soil.b. Mineral solubilisation or mobilization to be able to stimulate plant growth.
- 3.1.8. contain competent, persistent, and effective strains within its own agro-ecological conditions.
- 3.1.9. be effective and easy to apply and have adequate shelf life.
- 3.1.10. The manufacturing, use and even disposal of Azospirillum biofertilizer shall be in conformance to ES ISO 14000 and stipulated in the Environmental Protection Act of the laws of Ethiopia.

### 3.2. Specific Quality requirements

Table 3.2 : Specific Quality requirement for Azotobacter biofertilizers group

S.NO	Parameters	Azotobacter	Test methods
3.2.1	Base	Carrier based* or liquid based	A method in annex A or equivalent
3.2.2	Viable cell count 15 days after manufacturing	Minimum $10^7$ /g or $10^7$ /ml	
3.2.3	Viable cell count before 15 days of expiry	Minimum $10^6$ /g on dry mass basis or $10^6$ /ml	
3.2.4	Contamination level	No contamination at $10^{-5}$ dilution level	
3.2.5	PH	6.5-7.5	
3.2.6	Moisture % by weight (carrier based)	30-40%	
3.2.7	Efficiency character(min)	20% of fresh or dry biomass over the negative control	
3.2.8	Shelf life	6 months after the date of manufacture	

\*Type of carrier: The carrier materials such as peat, lignite, peat soil, humus, wood charcoal or similar material favouring growth of organism.

#### 4. Carrier materials specifications

4.1 Carrier materials for inoculants shall meet the following requirements:

1. The pH shall be readily adjusted to 6.5-7.0.
2. The carrier material shall have a high moisture-holding capacity (minimum of 20%)
3. The carrier shall be sterilized and free of lump forming
4. The carrier shall be free of toxic materials.
5. The carrier shall contain sufficient quantity of carbon, greater than 20%
6. The sterilized carrier shall be free of any contamination

#### 4.2. Particle size of carrier materials:

Azotobacter Carrier particle Shall pass through 150 micron sieve size.

#### 4.3. Packaging

##### 4.3.1. Packaging material

Biofertilizers shall be packed in double packaging plastic materials as it allow gas exchange. The outer packing low density polyethylene (75-100  $\mu$ m ) material shall be colored but not black where as the inner shall be transparent and high density polyethylene and steam sterlizable. If sterilization is done with radiation, single low density and opaque packaging plastic can be used. In principle the package system shall be easy to use and handle. More over, the containers, including packaging mateials, used to package biofertilizers shall be made only of substances, which are safe and suitable for their intended uses. They shall not impart any toxic substance or undesirable odor to the desired microorganism.

**4.3.2. Packaging size**

The net weight of inoculants per packet shall be manufactured at 125g amount for seeds that meant for a seed lot enough to cover quarter of hectare.

**5. Storage**

Biofertilizer shall be stored by the manufacturer in a cool and dry place away from direct heat preferably at a temperature of 15 °C to 30°C. It shall also be the duty of the manufacturer to instruct the retailers and, in turn, the users about the precautions to be taken during storage.

**6. Transportation**

Avoid direct sunlight, rain and temperatures shall not be less than 0°C and above 35°C during transportation.

**7. Labeling**

Labeling on the packaging bag, shall be according to the requirement of ES 3907-1.

**8. Sampling**

Sampling shall be according to the requirement in ES 3907-1.

## ANNEX A

**1. Carrier-based inoculants test**

For carrier-based inoculants, the qualities to be checked are:

1. pH
2. Moisture content
3. Viable number microorganism
4. Plant infection method (MPN)

**1.1. Determination of pH**

Inoculants shall possess neutral pH conditions. Monitoring of the inoculants pH condition shall be made regularly in the following way; Make 20 g of the inoculant suspension in 50 ml of distilled water and shake on a rotary shaker for 2 h, filter on Whatman No. 1 filter paper or equivalent under vacuum using a funnel. Determine pH of the filtrate in a pH meter at 25 °C.

**1.2 Moisture content**

Weigh to the nearest mg about 10 gm of the prepared sample in a weighed clean, dry Petri Dish. Dry in an oven at 100 °C -105 °C to constant weight. Cool in a desiccator and weigh. Report percentage loss in weight as moisture content.

Calculation:

$$\text{Moisture percent by weight} = \frac{100(B-C)\#}{B-A}$$

A = Weight of the Petri Dish

B = Weight of the Petri dish plus material before drying

C = Weight of the Petri Dish plus material after drying

**1.3 Viable number**

The number of viable microorganism is counted by spread-plate method. Serially dilute one gram of the inoculant to obtain dilutions of the order of  $10^{-5}$  to  $10^{-6}$ . Plate 0.1 ml aliquots of the dilutions on YEMA plates and incubate at  $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 3 to 7 days. The counts of viable microorganism in the inoculant shall be not less than  $10^7$  Cfu/g of inoculants dry mass. Otherwise, the lot of inoculant to which this sample belongs shall be rejected.

**1.4 Symbiotic effectiveness characterization**

Testing the symbiotic effectiveness of inoculants is extremely crucial as it clearly assures the biomass and nitrogen accumulation capacity of the target microorganism on the intended crop with relative to control under green house.

$$\% \text{ symbiotic effectiveness} = \frac{\text{Shoot dry weight of plants inoculated with test strain}}{\text{Shoot dry weight of plants supplied with Nitrogen}} \times 100$$

Annex C

Most Probable Numbers for use with 10 fold dilution and 5 tubes per dilution.

P <sub>1</sub>	P <sub>2</sub>	Most probable number for indicated values of P <sub>3</sub>					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.16	0.17
1	3	0.089	0.10	0.13	0.16	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.046	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16.0	--



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## **Annex 6: Standard operating procedure for testing the quality of phosphorus solubilising bacteria**

*Note: The standard operating procedure retained by the Ethiopian standards Agency has been used in this manual as an example. However, similar SOPs have been developed in other COMPRO-II countries. For training or application, the country-specific SOP will have to be used. However, for countries without their own SOP, they can customize this one. The list of reference includes the various materials used to compile the SOP.*

**ETHIOPIAN  
STANDARD**

**ES 3907-2: 2015**

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**Fertilizers-Biofertilizers-part 2:-Phosphate  
solubilizing microbial specification and test  
method**

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ICS: 65.080

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## Foreword

This Ethiopian Standard has been prepared under the direction of Technical Committee for **Fertilizer** (TC 15) and published by the Ethiopian Standards Agency (ESA).

The draft document (Working Draft, WD) has been submitted to the Secretariat by the Ethiopian Institute of Agricultural Research/EIAR/.

## References

During the preparation of this standard reference was made to the following documents:

*EAS 456: 2007- East African Organic Products Standard*

*The FNCA biofertilizer manual 2006*

*Indian bio fertilizer standards-*

*IS 8268:2001-Standard for Rhizobium*

*IS 9138:2002-standard for Azotobacter*

*IS 14806:2000-Standard for Azospirillum*

*IS 14807:2000-Standard for phosphate solubilizers*

Acknowledgement has been made to the said organization for their effort of presenting the draft document and for the assistance derived from the above sources.

## Introduction

Fertilizers directly increase soil fertility by adding nutrients. Biofertilizers add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing Phosphate or nutrient mobilization to stimulate plant growth through the synthesis of growth promoting substances. They can be grouped in different ways based on their nature and function. Most biofertilizers are produced from microorganisms such as *Rhizobium*, *Azotobacter*, *Azospirillum*, Phosphate solubilising bacteria. Other types include mycorrhizal biofertilizers, potassium mobilizing biofertilizers and zinc solubilising biofertilizers.

The use of biofertilizers offer economic and ecological benefits by way of soil health improvement and fertility. This standard is in line with the fertilizer policy of Ethiopia which aims to ensure availability of high quality biofertilizer products for efficient use by the farmers

Regulations on inoculant quality vary from country to country and no set of international standards exists. Brazil, Canada, France, and Uruguay have regulatory authorities supported by legislation. Australia, South Africa, and New Zealand have quality control programs in which inoculant manufacturers participate voluntarily. In many other countries such as USA and UK product quality standards are left to the discretion of the manufacturers. Whether legislatively set or internally established by the manufacturer, standards for inoculants products should be a compromise between theoretical possibilities and practical limitations. However, without defined standards quality control cannot work (Thompson, 1991a). There exists a consensus that the establishment of standards, whether voluntary or imposed, has improved legume inoculant quality.

The objective of this standard is to ensure that biofertilizers on the market are appropriately tested through the quality criteria provided while ensuring that farmers obtain only certified products and as well aid the industry in the manufacture of quality biofertilizers. This standard will also promote the safe use of biofertilizers and promote fair trade.

## Fertilizers-Biofertilizers-part 2:-Phosphate solubilizing microbial Specification and test method

### 1. Scope

This standard prescribes the requirements, method of sampling and tests for phosphate solubilizing biofertilizer.

### 2. Normative reference

The following referenced documents are indispensable for the application of this Ethiopian standard. Only the edition of the documents (including any amendments) shall be applicable

**ES 3907-1:-Fertilizers- Biofertilizers — part -1: *Rhizobium* Specification and test method**

**ES ISO 14001: Environmental management systems - requirements with guidance for use**

### 2. Terms and Definitions

For the purpose of this standard the definition in ES 3907-1 shall apply:

### 3.0. Requirements

#### 3.1 General requirements

##### 3.1. Phosphate solubilizing Biofertilizers shall:

- 3.1.1. contain competent, persistent and effective strain in minimum recommended population.
- 3.1.2. contain no more than the maximum allowed level of contamination.
- 3.1.3. have single or a combination of effective strains.
- 3.1.4. contain no pathogenic organisms that affect the biophysical environment.
- 3.1.5. contain carrier materials that are not harmful to the environment.
- 3.1.6. shall have the ability to solubilize phosphatic minerals
- 3.1.7. should also have characteristics of the ability to stimulate plant growth or other desirable functions to enhance tolerance to pest damage and amelioration of the soil.
- 3.1.8. contain competent, persistent, and effective phosphate solubilizing strains within its own agro-ecological conditions.
- 3.1.9. be effective and easy to apply, have adequate shelf life and free from any contamination.
- 3.1.10. The manufacturing, use and even disposal of phosphate solubilizing biofertilizer shall be in conformance to ES ISO 14000 and stipulated in the Environmental Protection Act of the laws of Ethiopia.

## 3.2. Specific Quality requirements

Table 3.2 : Specific Quality requirement for Phosphate solubilising biofertilizers group

S.No	Parameters	phosphate solubilizing bacteria	Test methods
		Carrier based*	A method in annex A or equivalent
3.2.1	Base	Carrier based*	A method in annex A or equivalent
3.2.2	Viable cell count 15 days after manufacturing	Minimum $10^7$ g of carrier material or $10^7$ /ml of liquid	
3.2.3	Viable cell count before 15 days of expiry	Minimum $10^6$ /g on dry mass basis or $10^6$ /ml	
3.2.4	Contamination level	No contamination at dilution	
3.2.5	pH	6.5-7.5	
3.2.6	Moisture % by weight (carrier based)	30-40%	
3.2.7	Shelf life	6 months after the date of manufacture	
3.2.8	P-solubilisation efficiency	30-50% capacity of tricalcium phosphate to $P_2O_5$	
3.2.9	p- solubilisation zone	1 mm diameter of Pikoviskiya media having tricalcium phosphate	

\*Type of carrier: The carrier materials such as peat, lignite, peat soil, humus, wood charcoal or similar material favoring growth of organism.

## 4. Carrier materials specifications

4.1 Carrier materials for inoculants shall meet the following requirements:

1. The pH shall be readily adjusted to 6.5-7.0.
2. The carrier material shall have a high moisture-holding capacity (minimum of 20%)
3. The carrier shall be sterilized and free of lump forming
4. The carrier shall be free of toxic materials.
5. The carrier shall contain sufficient quantity of carbon, greater than 20%
6. The sterilized carrier shall be free of any contamination

## 4.2. Particle size of carrier materials:

100 % phosphate solubilizing microbia Carrier material particle Shall pass through 150 micron sieve size.

## 4.3. Packaging

## 4.3.1. Packaging material

Biofertilizers shall be packed in double packaging plastic materials as it allow gas exchange. The outer packing low density polyethylene (75-100  $\mu$ m) material shall be colored but not black where as the inner shall be transparent and high density polyethylene and steam sterlizable. If sterilization is done with radiation, single low density and opaque packaging plastic can be used. In principle the package system shall be easy to use and handle. More over, the containers, including packaging materials, used to package biofertilizers shall be made only of substances, which are safe and suitable for their intended uses. They shall not impart any toxic substance or undesirable odor to the desired microorganism.

**4.3.2. Packaging size**

The net weight of inoculants per packet shall be manufactured at 125g amount for seeds that meant for a seed lot enough to cover quarter of hectare.

**5. Storage**

Biofertilizer shall be stored by the manufacturer in a cool and dry place away from direct heat preferably at a temperature of 15 °C to 30°C. It shall also be the duty of the manufacturer to instruct the retailers and, in turn, the users about the precautions to be taken during storage.

**6. Transportation**

Avoid direct sunlight, rain and temperatures shall not be less than 0°C and above 35°C during transportation.

**7. Labeling**

7.1. Labeling on the packaging bag, shall be according to the requirement of ES 3907-1.

**8. Sampling**

Sampling shall be according to the requirement in ES 3907-1.

## ANNEX A

**1. Carrier-based inoculants test**

For carrier-based inoculants, the qualities to be checked are:

1. pH
2. Moisture content
3. Viable number microorganism
4. Plant infection method (MPN)

**1.1. Determination of pH**

Inoculants shall possess neutral pH conditions. Monitoring of the inoculants pH condition shall be made regularly in the following way; Make 20 g of the inoculant suspension in 50 ml of distilled water and shake on a rotary shaker for 2 h, filter on Whatman No. 1 filter paper or equivalent under vacuum using a funnel. Determine pH of the filtrate in a pH meter at 25 °C.

**1.2 Moisture content**

Weigh to the nearest mg about 10 gm of the prepared sample in a weighed clean, dry Petri Dish. Dry in an oven at 100 °C -105°C to constant weight. Cool in a dessicator and weigh. Report percentage loss in weight as moisture content.

Calculation:

$$\text{Moisture percent by weight} = \frac{100(B-C)}{B-A}$$

A = Weight of the Petri Dish

B = Weight of the Petri dish plus material before drying

C = Weight of the Petri Dish plus material after drying

**1.3 Viable number**

The number of viable microorganism is counted by spread-plate method. Serially dilute one gram of the inoculant to obtain dilutions of the order of  $10^{-5}$  to  $10^{-6}$ . Plate 0.1 ml aliquots of the dilutions on YEMA plates and incubate at  $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 3 to 7 days. The counts of viable microorganism in the inoculant shall be not less than  $10^7$  CfU/g of inoculants dry mass. Otherwise, the lot of inoculant to which this sample belongs shall be rejected.

**1.4. Phosphate solubilization efficiency**

Phosphate solubilizers (PS) shall contain phosphate solubilising bacteria. Commercially produced PS bio fertilizers shall be certificated with guaranteed components such type of strains, microbial density, and biological activity.

**1.4.1 Determination of soluble phosphorus using Bray II method.**

1.5. P-solubilization zone shall be determined by Pikoviskaya method.



Annex C

Most Probable Numbers for use with 10 fold dilution and 5 tubes per dilution.

P <sub>1</sub>	P <sub>2</sub>	Most probable number for indicated values of P <sub>3</sub>					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.16	0.17
1	3	0.089	0.10	0.13	0.16	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.046	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16.0	--



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## **Annex 7: Guidelines for registration of bio-fertilizers**

*Note: The guidelines retained by the Tanzania Fertilizer Regulatory Authority (TFRA) for registration of bio-fertilizers have been used in this manual as an example. If your regulatory body has recommended a different format, please replace this version by the one recommended for your country. Stated differently, some sections should be customized to your country-specific requirements and situation. The list of reference includes the various materials used to compile the guidelines. When a specific excerpt is included the actual reference is added to it.*

### **1 INTRODUCTION**

In Tanzania, biofertilizers are currently captured in the Fertilizers Act under the definition of supplement. These guidelines for registration of biofertilizers in Tanzania are therefore developed to provide more guidance not only to applicants/registrants, but also to the staff of the Tanzania Fertilizer Regulatory Authority (TFRA) to ensure consistency in the registration process of biofertilizers. The implementation of the registration guidelines as well as applicable standard operational procedures (SOPs) for quality control will also enable the enforcement of compliance. Thus, biofertilizers intended for commercialization in Tanzania will have to comply with these guidelines to ensure product safety, quality, efficacy, and proper labelling.

Therefore, the key objectives of these registration guidelines are as follows:

- 1) To ensure that only registered biofertilizers are placed in the marketplace and made available for use by farmers
- 2) To ensure that only biofertilizers that are demonstrated to be of high quality, safe, and efficacious for intended use are registered
- 3) To ensure that the labels of registered biofertilizers contain adequate information for proper, safe use and handling of the products
- 4) To ensure that marketplace monitoring/surveillance after biofertilizer registration is institutionalized
- 5) To ensure that biofertilizers which were introduced in the Tanzanian marketplace prior to implementation of these guidelines are re-evaluated for conformity to the requirements outlined herein

The scope of these registration guidelines shall only cover biofertilizers containing naturally occurring microorganisms; in other words, microorganisms that have not been genetically engineered. For biofertilizers containing genetically modified organisms (GMOs), the TFRA shall direct the product proponents/potential registrant to the appropriate regulatory authority in Tanzania. Such an issue could be discussed during the pre-consultation meeting. Therefore, GMOs are out of the scope of these guidelines.

### **2 USE OF THE GUIDELINES**

The guidelines for the registration of biofertilizers have been developed by TFRA to ensure biofertilizers placed in the Tanzanian market are in compliance with relevant standards and regulations. In that regard the registration will ensure that biofertilizers in the market are efficacious for the intended purpose and safe to plants, animals, humans, and the environment

when used as directed. Registration of biofertilizers by TFRA will facilitate control and monitoring of these products by ensuring that only registered products are legally approved for sale and use in Tanzania. Therefore, these guidelines have been developed to provide registration procedures to be followed by TFRA and biofertilizer traders in Tanzania for regulation and market facilitation purposes. Availability of documented guidelines will provide transparency on regulation procedures and alignment of business plan accordingly before and during application for the registration.

### **3 SCOPE**

Registration guidelines shall only cover biofertilizers containing naturally occurring microorganisms that have not been genetically modified in any way, and in any other case shall be declared by the registrant or manufacturer.

### **4 REGISTRATION PROCEDURES**

#### **4.1 Pre-submission**

##### **4.1.1 Letter of intention**

When requesting a pre-submission meeting, a letter of intention should be addressed to the Chief Executive Officer (CEO) of TFRA. It will include at least a brief description (maximum 2 pages) of the intended use of the biofertilizer; the manufacturing company; the registrant's name and contact information; and the local agent's name and contact information when the registrant is not a Tanzanian resident. Equally important, the letter of intention should indicate whether the product has been approved for use in other countries in agricultural production. If it has not been used elsewhere, the letter should substantiate the reason. If at a later date the product proponent decides to apply for registration of the biofertilizer, the letter of intention will be transferred into the application package. The pre-consultation meeting is scheduled within 10 working days after the reception of the letter of intention, unless otherwise advised by the applicant.

##### **4.1.2 Consultation**

A pre-submission consultation meeting is proposed to provide guidance and advice to registrants prior to submission of an application package for registration. The pre-consultation meeting shall be face-to-face. Prior to or during the meeting, the following information shall be supplied in addition to the letter of intention:

- Details of the identity of the biofertilizer
- List of active agent(s)
- Tentative product marking and labelling information
- Proof of ownership of the biofertilizer to be registered
- Presentation of the research and development (R&D) data related to the efficacy of the biofertilizer (if available)
- Brief rationale of product safety and quality (maximum 2 pages)

The discussion is intended to ensure that the registrant submits a comprehensive application package for the biofertilizer registration. TFRA will answer questions of the registrant and

clarify any issues related to the registration guidelines, quality control criteria, or regulatory requirements.

#### **4.1.3 Feedback to applicants on the pre-submission meeting**

After the consultation meeting, TFRA shall send a summary of the discussion and feedback on any issues that have not been adequately addressed during the discussion (e.g., information from an external source). The summary shall include a comprehensive list of items required for the registration process. TFRA's feedback shall be sent in writing to the registrant within 20 working days to the physical address indicated in the letter of intention and using registered mail. Equally important, the feedback shall outline the way forward including, but not limited to, the following information:

- i. Confirmation that the applicant is a suitable registrant as being the owner of the biofertilizer or in a suitable relationship with the owner based on information provided during the pre-consultation meeting.
- ii. Authorization to apply for the product registration by submitting all the required items in a single package.
- iii. A comprehensive list of the safety, efficacy, and marking and labelling requirements.
- iv. Confirmation of data waivers if applicable (e.g., in case of equivalence of data).
- v. Information on the handling procedures of confidential business information (CBI).
- vi. Details on the required fees to be paid along with submission as well as the fee structure including annual fee and other costs (i.e. cost of reviews of the label, efficacy, quality, and safety data in the application package; cost of the quality control; and cost of efficacy trials).
- vii. Other administrative requirements as applicable.
- viii. When, based on information provided, the product requires review by multiple registration authorities (e.g., TFRA and the Tropical Pesticides Regulatory Institute [TPRI]), an explanation of the applicable procedures.
- ix. Expected timeframe of application reviews.

## **4.2 Application procedures for registration of a bio-fertilizer**

### **4.2.1 Administrative requirements**

The administrative requirements are meant to provide applicants with enough information to prepare the registration package based on the regulatory requirements and procedures. *Annex 1* includes details of the checklist to be used. The following outlines the minimum items required for the application package for biofertilizer registration.

#### **4.2.1.1 Application form**

Applicants shall be required to complete an application form (*Annex 2*) by providing a summary of the information about the product as described in the application form.

#### **4.2.1.2 Registration fees**

The registration fee includes document/data review fees, payable to TFRA. It doesn't include the cost for factory inspection, efficacy and safety data generation, and testing for compliance to standards; these payments should be paid directly to the institutions/organizations generating the data and they are not controlled by TFRA. TFRA only specifies the minimum data required; however, the efficacy and quality data should be generated by an institution approved by TFRA. The fee structure applied by TFRA for bio-fertilizers registration is available on request.

#### **4.2.1.3 Authorized representatives**

To ensure that confidential business information (CBI) is not released to unauthorized individuals, the applicant should provide in writing a list of maximum three persons authorized to request information on the product file when necessary. Details on the individuals including their contact information, physical address, and their relationship with the product proponent should be specified. Any other person who is TFRA-authorized staff and who is not included in the list of the applicant shall not have access to information in the product file or details related to the registration process for the specific biofertilizer. The applicant can modify the list at any time in writing. However, TFRA will not be responsible for any damage that may occur during the transition period, i.e. from the initial list to the revised list of authorized individuals. It is the responsibility of the applicant to ensure that TFRA receives the correct and valid information on time. Once the amended list is received TFRA shall immediately implement the change.

#### **4.2.1.4 Declaration of local agent**

When an applicant is not a citizen of Tanzania, he/she must have a Tanzanian citizen as local representative or agent (also known as country representative). An official letter signed by the product proponent and countersigned by the local agent should be submitted to TFRA. The local agent must be included in the list of authorized representative(s).

#### **4.2.2 Efficacy requirements**

In addition to the administrative requirements, the application package should include efficacy data generated by an institution/organization approved by TFRA. The efficacy data shall include performance data generated based on the requirements in these registration guidelines. A summary of the efficacy data (maximum 5 pages) should be submitted alongside any other relevant supporting data.

Upon reviewing the nature of guarantees or claims displayed on a product label, TFRA shall determine the type of efficacy data to be submitted. Similarly, if the efficacy of a given use-pattern is not well established TFRA may request efficacy data to substantiate the recommended use-pattern. Stated differently, each usage pattern or direction for use on the product label is treated as a claim, and must be supported by scientifically valid efficacy data. Anecdotal or testimonial evidence will not be accepted. Below are the minimum requirements for efficacy data; however, TFRA reserves its rights to requested additional information when deemed necessary.

#### **4.2.2.1 Good Quality System Procedures**

It is recommended that efficacy trials be conducted using Laboratory Quality System Procedures (LQSP; also known as Good Experimental Practices or GEP). The primary goal of LQSP (*Annex 3*) is to ensure that all efficacy trials conducted are of high quality and the results derived are reliable enough to support the registration of a biofertilizer. This is particularly important in the context of mutual recognition across countries. Hence, only institutions/organizations that can demonstrate adequate LQSP will be approved by TFRA to generate efficacy data for product registration. Importantly, the approval duration of the institutions/organizations and research scientists shall be subjected to renewal to ensure the sustainability of the LQSP.

#### **4.2.2.2 Location of efficacy testing and site selection**

The location of efficacy testing shall be recommended by TFRA. Hence, TFRA shall determine the minimum number of agro-ecological zones (AEZ) where a given biofertilizer should be tested. However, the site selection within the recommended AEZ is left at the discretion of the approved institutions/organizations. The AEZ choice shall also be based on the agricultural production regions for the intended crop(s). The minimum AEZ number shall also depend on where the applicant wants to sell or distribute the product of interest.

*In all instances, the efficacy data shall be generated in an environment relevant for the intended use. For instance, greenhouse trials will not be enough to support the efficacy of a biofertilizer intended for field use.*

#### **4.2.2.3 Minimum number of trials**

The minimum number of trials required to support product efficacy will vary, depending for instance on the intended usage pattern, intended crops, and label claims. For example, a product intended for soil application, to be used on a single crop with the claim of increased yield, would generally require a low number of trials. However, when the same product is intended for several crops, and the biofertilizer label includes several claims in addition to yield increases, the minimum number of trials may significantly increase. In general, the minimum number of trials/sites is set to 6 when the various claims could be tested at the same time at the 6 sites.

#### **4.2.2.4 Spatial and temporal distribution of the minimum number of trials**

In general, the minimum number of trials should be conducted during more than one growing/cropping season (temporal variability) and in different AEZ or trial sites (spatial variability). The minimum number of growing seasons shall be determined by TFRA and communicated to the registrant in the feedback letter following the pre-submission meeting.

When agreed upon during the pre-consultation meeting, TFRA may grant a waiver for the temporal variability, but shall improve on the spatial variability (i.e. accelerated trials). As such, the minimum number of trials shall be increased as well as the number of AEZ or trial sites. For instance, when the minimum number of trials capturing the spatial and temporal variability is 6, then the minimum number of accelerated trials shall be 9 trial-sites in various AEZ.

#### **4.2.2.5 Biofertilizer recommended for several crops**

When a biofertilizer is recommended for several crops, the following two options shall be discussed during the pre-consultation meeting to optimize the number of trial sites:

- 1) Representative crops: In the case where the similarities between crops is supported by scientific evidence in terms of physiology and production patterns, the crops may be grouped together based on similarities and the efficacy testing conducted using representative crops from each group. The grouping shall have to be approved by TFRA before the beginning of the trials. Grouping will not be considered in the case of few crops (e.g., less than 3 crops).
- 2) Representative site: When possible, the effect of the biofertilizer on the selected crops shall be evaluated at the same sites keeping the same minimum number of trials for each crop.

TFRA reserves the right to determine the most appropriate option for a given biofertilizer and crop(s); the decision will be made on a case-by-case basis.

*In all instances, if the biofertilizer is tested on a reduced number of crops compared to the list on the product label, the label will have to specify the crop species that were used to generate the efficacy data.*

#### **4.2.2.6 Trial design**

A randomized complete block design in which the treatment plots are randomly distributed within each block is recommended. Other designs such as the completely randomized design/plan may be used for trials conducted in completely homogeneous environments, such as greenhouses or growth chambers. There is a general consensus that the trials should also be



designed in a manner that allows at least 10 degrees of freedom to the error term during the analysis of variance (ANOVA).

#### **4.2.2.7 Preventing cross-contamination between experimental plots**

Guard rows or border plots to prevent treatment overlap and/or drift of product or treatment from one plot to another are recommended. Guard rows are particularly important when the plot size is small. Plot size will vary depending on the crop of interest, the product, and the method of application of the product. The plot size will be discussed during the pre-consultation meeting.

#### **4.2.2.8 Treatment structure**

Treatments with the biofertilizer intended for registration must be compared to one or more check treatments, including but not limited to (a) an untreated control and (b) appropriate comparative treatments (positive controls; for instance a registered biofertilizer intended for the same use and with the same mode of action).

#### **4.2.2.9 Planting window**

When the product label does not recommend to sow the seeds treated with the biofertilizer immediately after treatment, the label should specify the timeframe that is allowed between the treatment and planting. Such a timeframe is known as the planting window. The planting window should be substantiated using scientific data to ensure that the required number of viable cells in the biofertilizer of interest can be recovered from the seeds at the end of the planting window. The planting window shall be demonstrated not only based on laboratory data (counting), but also field data obtained when the product is tested at the end of the planting window. A tolerance of one tenth of the recommended planting window will be tolerated; however, the tolerance won't exceed 7 days when the planting window is significantly long.

#### **4.2.2.10 Equivalence of data**

When data collected in other countries are found to be relevant in Tanzania based on the similarity of the agro-climatic conditions, TFRA will apply the principle of equivalence data, also known as mutual recognition. In such a case, TFRA will require a reduced number of trials (half of the minimum number of trials generally required and in a single growing season) to confirm the efficacy of the product under the local conditions. The equivalence of data shall be granted based on the merit of the rationale and evidence provided to support the similarity of the agro-climatic conditions.

#### **4.2.2.11 Expiry date and shelf life for biofertilizers**

The active ingredients in biofertilizer products are subjected to degradation or loss of viability over time. A biofertilizer label shall display an expiry date, i.e. the date after which the required minimum number of viable cells can no longer be guaranteed. Alternatively, the

product label shall display the manufacturing date and the shelf life; the shelf life represents the expected storage duration (from the manufacturing date) within which the required minimum number of viable cells can be guaranteed when the biofertilizer is stored under the recommended conditions.

#### 4.2.2.12 Measurable parameters

When evaluating the efficacy of a biofertilizer, data should be collected before planting, during the growing season, and at harvesting. TFRA will discuss with the applicant the minimum measurable parameters required, as this is a critical determinant in the registration process.

- a) **Before planting:** To ensure that product efficacy is evaluated in appropriate conditions, the initial soil fertility level should be evaluated prior to the establishment of the trials. Soil analysis prior to treatment should include (but is not limited to): soil texture, pH, cation-exchange capacity, organic matter content, availability of selected plant nutrients, functional microorganisms of interest (in this case of biofertilizers), etc. This information should be collected regardless of the label claims.
  
- b) **During the growing season:** After the application of the treatment, minimum weather data including the ambient temperature, humidity, and rainfall should be collected. In addition to the initial soil testing, information on weather conditions could also be used to explain any spatial or temporal variability. Other measurable parameters shall be based on the label claims, but don't have to be limited to the label claims. Measurable parameters will include, but are not limited to, the following:
  - 1) Visual symptoms of nutrient, abiotic, and biotic stress (when observed)
  - 2) Pod numbers for legumes
  - 3) Uptake of plant nutrients of interest at a specific growth stage
  - 4) Number, weight, and colour of nodules in the case of rhizobium inoculants
  - 5) Intensity of root colonization/infection, and root and hyphal length in the case of mycorrhizal inoculants
  - 6) Root architecture in the case of plant growth promoting rhizobacteria
  
- c) **At harvesting:** The following measurement parameters should be considered. Crop yield remains by far the most critical parameter.
  - 1) Tissue analysis for nutrient uptake (i.e. major, secondary, and micro- nutrients depending on the product of interest)
  - 2) Crop yields (particularly grain yield when applicable, haulms, husks, etc.)
  - 3) Quality of the crop yields when applicable (based on label claim)

#### 4.2.2.13 Data analysis

The data should be statistically analysed, including descriptive statistics and analysis of variance (ANOVA) using accepted software (e.g., SAS, R). The method of statistical analysis should be considered prior to conducting the trial. The level of significance and the mean separation techniques should be the ones that are commonly used in the field of interest (e.g., probability of 5% and the least significant difference i.e. LSD). Anecdotal or testimonial

evidence should not be considered as a scientifically valid form of efficacy data, and therefore should not be accepted to support product approval for registration; however, such evidence could be used as background information. Hence, all label claims must be adequately supported by scientific data that has been statistically analysed and demonstrates a statistically significant benefit. The statistical analysis should be consistent across AEZ for the same product and treatments.

#### 4.2.2.14 Profitability analysis

Economic evaluation of crop responses to biofertilizers is important to assess their profitability, particularly in the context of smallholder farmers. The benefit cost ratio (BCR) is one of the tools used to conduct such an evaluation, particularly when yield is considered. BCR is the ratio of the net value of extra crop produced to the cost of the biofertilizer (Eq. 1). In actual practices, a BCR of 2.5 and above is considered to be satisfactory for the adoption of the technology. However, some authors have indicated that a  $BCR > 1$  is already attractive for farmers. Taking into consideration other costs, including credits and risk taken, the higher the BCR the better. For the purpose of biofertilizer registration, TFRA shall accept BCR values  $\geq 1$ . Such BCR values shall be shown in at least 60% of the trial sites for a biofertilizer to be registered by TFRA.

$$BCR = \frac{\text{Value of additional crop produced} - \text{cost of the biofertilizer}}{\text{Cost of the biofertilizer}} \quad (\text{Eq. 1})$$

#### 4.2.3 Safety requirements

A package of safety data should be prepared. In addition to the supporting document(s), a rationale not exceeding 5 pages to substantiate the safety of the biofertilizer should be provided. Basically, the applicant shall demonstrate that each ingredient in the product formulation is present at a level expected to be safe to humans, animals, plants, and the environment when used as directed. Also, the safety of the formulation when all the ingredients are mixed together should be demonstrated i.e. mixing the ingredients results in a product equally safe. Equally important, the level of non-guaranteed micro-organisms in the biofertilizer product should not be higher than the tolerance prescribed in the quality-control standard-operational-procedure applicable to the specific product. TFRA will keep a database of approved SOPs to be used for quality control of biofertilizers sold in Tanzania. No pathogen micro-organism or ingredient will be tolerated in a biofertilizer intended for sale in Tanzania. TFRA reserves the right to ask for additional safety information on the biofertilizer not only during the registration process, but also during the post-registration period when deemed necessary. The level of contamination of biofertilizers will be evaluated by laboratories approved by TFRA.

The requirements to establish the safety of a biofertilizer include, but are not limited to, the following:

1. Summary (5 pages maximum) of the technical dossier related to the safety of the product (i.e. bio-safety assurance statement on pathogenicity, gene flow, invasiveness, persistence, and effect on non-target organisms, among others)
2. Evidence (i.e. data and/or biographical references) supporting the safety of the biofertilizer
3. Information on the safety of the carrier material (when applicable)
4. Manufacturing process
5. Quality assurance (QA) and quality control (QC) procedures of the manufacturing plant (i.e. company self-evaluation on quality)
6. Information on the strain(s) including the source, identification, and taxonomy
7. Declaration that the biofertilizer does not contain GMO ingredients
8. Material safety data sheet (MSDS) of the biofertilizer and ingredients (when available)
9. Any additional information and supporting documents or items deemed necessary by the applicant or TFRA

#### **4.2.4 Labeling and packaging requirements**

##### **4.2.4.1 Product label**

The applicant should provide a proposed tentative product label to allow correct use of the biofertilizer during the efficacy testing and its safe handling. Based on the efficacy and safety data, the label will have to be amended accordingly into a commercial label. The commercial label will also have to include a registration number granted by TFRA as a seal of approval. Equally important, the label of a biofertilizer sold in Tanzania shall be at least in both English and Swahili.

Product labels shall not have any incorrect or misleading information, mark, brand, or name that would tend to deceive or mislead the end-user with respect to the composition or benefits of using the product. The approved product label shall be the only commercial label found in the marketplace for the registered biofertilizer.

##### **4.2.4.2 Specific requirements for labelling and packaging**

Any of the items below that is not qualified as optional is by default mandatory.

1. Product name
2. Guarantee analysis: a statement that shows the content (concentration of the active ingredients) of the product, i.e. CFU of active ingredient(s) per gram of finished biofertilizer product. TFRA will verify the guarantee analysis using the service of laboratories approved by TFRA for quality control of biofertilizers
3. Directions for use: specifying application rates, frequency of application, target crops, application equipment, and method of application, among others (the directions for use should be based on the results of the efficacy trials)
4. Registration number: seal of approval by TFRA

5. Batch number
6. Manufacturing date
7. Expiry date or shelf life
8. Company information: contact address including the physical address of the place of manufacture
9. Net weight using the International System (IS) of units of measure
10. Cautionary statement (safety statement)
11. Planting window or pre-planting treatment period (when applicable)
12. Storage conditions
13. Handling conditions
14. Disposal statement for both the biofertilizer and containers
15. Brand name (optional)
16. Net weight in local equivalence of the IS units of measure (optional)
17. Seller's guarantee: warranty statement in case of damage or product failure when product is used as per the direction for use (optional)
18. Label claims (optional)
19. List of ingredients (optional)
20. Website (optional)

#### 4.2.5 Quality requirements

Biofertilizer quality is one of the most important factors resulting in its success or failure. The SOPs for the quality control of biofertilizers in Tanzania (available on request) will provide detailed quality criteria of specific biofertilizers. Items considered for the quality of biofertilizers sold in Tanzania include, but are not limited to, the following:

- 1) Viable cell count for bacteria based products (i.e. CFU per gram of finished biofertilizer product)
- 2) Propagules per gram of finished biofertilizer product for arbuscular mycorrhizae fungi (AMF) based products
- 3) Contamination data at a specific dilution level
- 4) pH of the biofertilizer product
- 5) Efficacy character of the biofertilizer (i.e. quick test of the potential efficacy of the product in controlled environment conditions. This shall not be considered as evidence of field performance)
- 6) Moisture content (Note: limited to solid formulations, i.e. biofertilizers in granular or powder form)
- 7) Particle size (Note: limited to biofertilizers in powder form)

*When TFRA does not have a specific SOP to verify the quality of a novel biofertilizer, the applicant will have to provide the procedure to TFRA. TFRA shall validate it in its approved laboratories before the biofertilizer can be registered.*

#### 4.2.6 Sampling requirements for quality control

Product sample shall be significantly representative of the biofertilizer batches. Adequate precautions shall be taken to prevent contamination of the biofertilizer during the sampling process.

#### **4.2.6.1 General requirements for sampling**

- 1) Precautions and directions must be observed when drawing, preparing, and handling samples intended for quality control
- 2) To ensure that representative samples are taken, sampling must be carried out by a qualified, trained and experienced inspector
- 3) For packaged products, unopened packets should be sampled and sent to the laboratory using appropriate equipment to prevent possible contamination of samples during handling
- 4) Samples must be taken in the presence of the product proponent or his/her authorized representative
- 5) Collected samples should not show any visible sign of contamination
- 6) For quality control, samples shall be taken from representative lots in a random manner
- 7) Adequate and representative samples for testing should be taken. The product proponent shall retain one unit as a representative sample for re-testing in the case of dispute

#### **4.2.6.2 Drawing samples**

- a) The number of sample-packets to be taken from a lot will depend on the size of the lot. The samples should be randomly selected using a well-established randomization procedure. The sample will be considered a legal sample as the result will be used as a proof of the product compliance to the regulatory requirements.
- b) The inspector or any other authorized staff shall take 9 sample-packets from a given lot based on the batch number (3 of the samples will be sent to the laboratory approved by TFRA for quality control; 3 will be handed to the product proponent in case he/she may want to send them to a reference laboratory to cross-validate the result from the approved laboratory; and the remaining 3 will be kept in adequate conditions for re-testing in case of conflicting results or dispute). The product proponent or his/her authorized representative shall sign onto the sampling form of the inspector. When analysing samples, each packet should be analysed separately.
- c) The inspector or any other authorized staff should carry with him/her appropriate sampling forms on which to consign information about the sample and the sampling procedures. He/she should ensure to have appropriate sampling tools for taking and transporting samples to the laboratory. Each sample shall be sealed in cloth bags or any approved containers using the inspector seal after having put inside the sampling form filled out by him/herself and signed by the product proponent.
- d) The total number of lots to be sampled will depend on how many there are. When the total number of lots is lower than or equal to 5, each of them will be sampled. When it is higher than 5, 5 randomly selected lots will be sampled. The minimum number of samples per lot shall be as shown in the table below:

Packets per lot	Number of samples
≤5,000	03
>5,000 - ≤10,000	04
> 10,000	05

Note: number of randomly selected samples to be drawn from a lot

### 4.3 Feedback to the applicant on the registration process

When the registration process is completed, TFRA will communicate the result in writing to the applicant including a rationale of the decision and information on the post-registration requirements. When all the efficacy and safety data have been received and a commercial label provided, TFRA will make a decision related to the registration of the biofertilizer within a period of three months.

#### 4.3.1 Registration decision

Based on the evidence provided, TFRA will take one of the 3 potential decisions below:

- 1) Full registration (FR) of the biofertilizer
- 2) Conditional registration (CR) of the biofertilizer (note: the conditions will be specified in the decision letter)
- 3) Denial of the registration of the biofertilizer (note: a rationale will be included in the decision letter)

All registered biofertilizer products will be subject to renewal of the registration (the application form will be available on request) as follows.

- 1) In the case of conditional registration, the registrant will have to fulfil the requirements for full registration within six months. Failure to do so will lead to the registration being denied. There will be no room for renewal of conditional registration. In the case of full registration, upon continued compliance with the registration procedures and regulations, the registrant shall apply for the renewal of the registration every 3 years.

#### 4.3.2 Documentation of registration decisions

Once a decision is taken it will be recorded in the official Register. Hence, each biofertilizer registered will be assigned a specific registration number. The registration number will have to be included on the registration certificate as well as on the commercial product label. The registration number for conditional registration should include the letters “CR”, whereas that of full registration will include “FR”. The registration numbers will read as follows:

- 1) Registration number: TFRA DDMMYYYYBFCRXX or
- 2) Registration number: TFRA DDMMYYYYBFFRXX

Where DD stand for the day, MM for the month, YYYY for the year, BF for biofertilizer, CR for conditional registration, FR for full registration, and XX for a number from 01 to 99 in the precedence order of registration.

The history of a given product registration process should be kept in the Register. The Register of biofertilizers will be in electronic form; however, hard copy will be kept for a minimum of 3 years from the date of the registration decision. The renewal of the registration will not affect the registration number; a registered biofertilizer will keep the same registration number during its entire lifetime in the Tanzanian marketplace.

#### **4.3.3 Certificate of Registration**

When a biofertilizer is registered, the registrant shall get a registration certificate including, but not limited to, the following information:

- 1) Name of the registrant and local representative if it is not the same person
- 2) Company's name and physical address of the place of manufacturing of the biofertilizer
- 3) Name of the biofertilizer
- 4) Registration number
- 5) Type of registration (full or conditional; also reflected in the registration number)
- 6) Period of validity
- 7) TFRA's seal (i.e. seal of approval)
- 8) Full name and signature of the CEO of TFRA
- 9) Date of registration or registration renewal

#### **4.3.4 Appeal of a registration decision**

When applicant/registrant is not satisfied by a decision made by TFRA regarding the registration status of a biofertilizer, he/she has the right to appeal according to the Fertilizer Act of Tanzania. TFRA will provide the applicant with adequate guidance related to the appeal procedures and instances.

### **5 Concluding remarks**

The TFRA registration guidelines for biofertilizers outline the various requirements for the attention of TFRA staff and product proponents. They allow for a transparent, fair, and timely review of applications for biofertilizer registration in Tanzania. All biofertilizers intended for sale in the Tanzanian market shall comply with these registration guidelines. Any biofertilizer product found in the Tanzanian market without the appropriate registration certificate and the related registration number shall be subject to enforcement including potential detention. For products that were found in the market prior to the implementation of these registration



guidelines, the product proponents are required to contact TFRA to agree on the appropriate actions to make those products compliant to this biofertilizer regulatory framework.

Both TFRA staff (including regulatory officers, inspectors, and laboratory staff involved in the quality control of biofertilizers) and product proponents are required to familiarize themselves with these guidelines. The quality control requirements (labelling, efficacy, quality, and safety data) are not limited to the registration process, but also apply to post-registration marketplace monitoring. Product proponents are required to ensure that the quality of the biofertilizers in the marketplace is up to the standards prescribed in the registration guidelines or applicable standard operational procedures for quality control, which are available on request. Stated differently, if a product in the marketplace no longer meets the quality standards (labelling, efficacy, and safety requirements), it will be subjected to enforcement actions including (but not limited to) detention or cancelation of the registration certificate.

To ensure timely access to innovative technologies by Tanzanian farmers, TFRA will clarify the file review timeframe during the pre-submission meeting. Once all the required items are submitted, TFRA will process the file in a timely manner. To prevent unnecessary delay, applicants are required to provide TFRA with any clarification or item required at their earliest convenience. Hence, the registration process is interactive. Both parties (i.e. TFRA and the applicant) will have to take advantage of it. However, the registrant shall follow up with TFRA for the progress of the registration process only when the timeframe indicated in the feedback of the pre-submission meeting has elapsed.

After the registration of a biofertilizer by TFRA, the registrant can commence legal sale of the product in the Tanzanian marketplace. He/she is also responsible for the quality of the product in the marketplace (commercial chain), unless there is significant evidence that the product has not been handled as prescribed on the product label by a third party (e.g., retailer). For instance, the product label shall clearly indicate that biofertilizer repackaging by unauthorized persons or adulteration is prohibited. Importantly, once a biofertilizer is registered, the registrant shall not make any modification to the product label or product formulation (ingredients and their proportion in the product) without a written approval from TFRA. Such changes represent a modification and will be reviewed by TFRA to evaluate the impact on the product registration status.

When the validity of a registration is running out and the registrant wishes to continue marketing and selling the product, the registrant has to apply for the renewal of the registration certificate using the recommended application form (available on request). He/she will have to indicate in writing whether he/she has the intention of modifying the product label or formulation. If no change is expected, no additional information will be required except the application form. If changes are expected, the registrant has to provide details of the changes in writing. The information will be reviewed by TFRA. TFRA also reserves the right to require additional information including quality, efficacy, and/or safety data depending on the nature of the changes. Registrants are required to submit the renewal request of the registration certificate at least 4 months prior to the expiry date of the current certificate.

When a biofertilizer fails to comply with the regulatory framework after registration, it will be subject to enforcement actions that may include, but are not limited to, product detention or cancelation of the registration certificate. The situation could result either from a contravention by the registrant to a prescribed standard, or emergence of new data on the efficacy, quality, or safety of the biofertilizer. When a biofertilizer is detained or registration certificate cancelled, TFRA will inform the product proponent or registrant in writing; the letter will include the reasons of the enforcement action and the requirements to address the issues. Detained biofertilizers will only be released when all the issues have been addressed. The same applies to the reactivation of the registration certificate; a newly signed registration certificate will have to be granted at his/her charge. If the product proponent or registrant cannot address the issues related to the detention of a product or cancelation of a registration certificate within the timeframe agreed upon with TFRA, the products will be disposed at his/her charge.

As a result of the implementation of the registration guidelines, TFRA has started developing various SOPs intended to provide more details or clarification on the enforcement of the biofertilizer regulatory framework, including the fee structure, penalties on violation of regulations and procedures, and dispute settlement. Equally importantly, when a registrant requires clarification on a specific provision of these guidelines, he/she is required to contact TFRA for that effect. This also applies to the appeal process for any decision made by TFRA under the Fertilizer Act.

## 6 GLOSSARY OF TERMS

**Lot:** All packets/containers of a product material from the same batch of manufacture in a consignment. Collection of the same materials of the size and style which have been processed under the same condition.

**Batch** - Specific quantity of materials (inoculant/ biofertilizer) manufactured in a single operation

**Batch number** – a combination of numerals and/or letters used to identify material pertaining to a particular batch and serving to distinguish it from all other batches of like materials

**Biofertilizer:** a substance which contains living microorganisms which colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of nutrient and/or growth stimulus to the target crop, when applied to seed, plant surfaces, or soil.

**Active ingredient:** an ingredient in a biofertilizer to which plant growth activity is attributed.

**Genetically-modified organism:** any living organism that possesses a novel combination of genetic material obtained from another organism through the use of modern biotechnology.

## 7 References

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## **Annex 8: Training materials for biofertilizer Inspectors**

*Note: This generic content should be customized based on the specific regulatory framework in each country*

### **1 Executive summary**

This manual provides guidance to inspectors conducting bio-fertilizer inspections or surveillance for conformity to the fertilizers regulations. It is intended to outline the key role and responsibilities of an inspector under the existing Ghanaian regulatory frameworks.

Chapter one covers the introduction where the aim and scope of the manual are elucidated. Also the key technical terms used in the manual are defined. Chapter two covers the fundamentals of Bio-fertilizer inspection such as: the need for due professional judgment, inspection Principles, check list prior to inspection, procedure of conducting inspection, Etiquette of the Inspectors, standards of professional conduct. Safety issues, Powers and Responsibilities of Bio-fertilizer Inspector, Warehouse for Storage of Bio-fertilizer and Reporting are well covered. Concluding this chapter are bio-fertilizer dealer related information which the inspectors should endeavour to enlighten them about, i.e. significance and importance of dealing in registered bio-fertilizer and Inspection Obligations of a Bio-fertilizer Dealer. Chapter three briefly touches on Bio-fertilizers regulations and registration guidelines while Chapter 4 is on Labelling requirements where Specific requirements for labelling and packaging are highlighted. Concluding the manual is Chapter five which covers sampling and handling of products.

This Inspection Manual is not a regulation and, therefore, does not add, eliminate or change any existing regulatory requirements. The statements in this document are intended solely as guidance. This document is not intended, nor can it be relied on, to create any rights enforceable by any party in litigation in Ghana.

### **2 Introduction**

#### **2.1 Introduction to the Manual**

This manual provides guidance to inspectors conducting bio-fertilizer inspections or surveillance for conformity to the fertilizers regulations. It includes recommended procedures and forms for the inspections.

Inspections are conducted in four phases:

- i) inspection preparation;
- ii) observations and evidence collection;
- iii) receipt for samples/statement(s), and analysing data/information; and
- iv) Inspection reporting.

Conducting all inspections in this manner will ensure that the inspector fulfils the statutory requirements for conducting inspections authorized under the governing regulations. The regulatory agency develops forms for use during inspections that will assist the inspector in completing all phases.

#### **2.2 Aim**

This Inspectors training Manual is an inspection support tool provided for use by inspectors from different countries while conducting bio-fertilizer inspections under Ghanaian regulations.

### 2.3 Scope of the Manual

This Manual is intended to outline the key role and responsibilities of an inspector under the existing regulatory framework. As inspectors perform their responsibilities, new lessons may be learned. Hence, it should be considered as a living document that should be updated as new knowledge is gained.

### 2.4 Key technical terms as used in this manual

**Active ingredient:** the micro-organism and any associated metabolites in a bio-fertilizer to which plant growth activity is attributed.

**Batch:** All inoculants that are prepared from a batch of fermentor or a group of flasks run at the same time and during the same timeframe.

**Batch number:** Is a specific number used to distinguish each batch. The number could be combination of symbols including letters and figures. The number should at least include the date of manufacture (dd/mm/yyyy). The number should be printed on each product package.

**Beneficial organism** any organism directly or indirectly advantageous to plants, or plant products, including biological control agents;

**Bio-fertilizer** a preparation or substance containing living organisms which colonize or are intended to colonize the rhizosphere or the interior of the plant that helps or enhances plants to take up nutrients or solubilize or mobilize soil nutrients;

**Indigenous microorganism:** a microorganism originating naturally in the country or region in which the bio-fertilizer is being registered.

**Inspector** a person authorized by a National bio-fertilizer regulatory body to discharge its functions

**Local agent** a person who is registered and legally operating in a given country;

**Lot** All packets/containers of a product material from the same batch of manufacture in a single consignment.

**Non-indigenous microorganism** a microorganism originating from outside the country or region in which the bio-fertilizer is being registered.

**Regulated article** any plant, plant product, storage place, packaging, conveyance, container, soil and any other organism, object or material capable of harbouring or spreading pests, deemed to require phytosanitary measures, particularly where international transportation is involved

**Inspection:** a process of examination of a product design, product, service, process or plant and determination of their conformity with specific requirements or on the basis of professional judgment or general requirement.

**Sampling:** Process of selecting a small quantity which is a most representative to the original large stock.

**Sample** Representative fraction of material for testing or analysis in order to determine the nature, composition, quality, and percentage of specified constituents.

## **3 Bio-fertilizer inspection**

### **3.1 Principles and Techniques of Fertilizer Inspection**

#### **3.1.1 Inspection**

Due professional judgment should be used in planning and performing bio-fertilizer inspections and in reporting the results. Inspectors are required to exercise reasonable care and diligence and to observe the principles of serving the public interest and maintaining the highest degree of integrity, objectivity, and independence in applying professional judgment to all aspects of their work.

#### **3.1.2 Due professional judgment**

Due professional judgment requires that the inspector(s): -

- ✚ Employs professional and organizational practices in accordance with all applicable laws, rules and regulations as per Ghana regulatory requirement,
- ✚ Conducts inspections in a timely, diligent and complete manner, using appropriate methods and techniques,
- ✚ Gathers evidence and reports in a fair, unbiased and independent manner and report findings, conclusions and recommendations are valid and supported by adequate documentation,
- ✚ Ensures that at all times, the action of inspection conforms to high standards of conduct, including adherence with the inspection ethics

#### **3.1.3 Inspection Principles**

The following principles should prevail during inspection:

- ✚ Impartiality: neutrality must be observed,
- ✚ Inspector must observe independence, accuracy, openness and fairness,
- ✚ The inspector must be free from commercial, financial and other pressure which might affect his judgment or place him/her in a conflict of interest,
- ✚ Inspection procedures must be applied in a strict manner and if sampling is to be done then a sample obtained is accurate and representative of the consignment,
- ✚ Professional judgment also should be applied when actually performing inspection and when evaluating and reporting the results of the work.
- ✚ In conducting an inspection, inspectors may employ the methods of inquiry most appropriate for the object of the assessment.
- ✚ Professional judgment requires inspectors to exercise professional skepticism, e.g., questioning and critically assessing evidence, throughout the inspection.
- ✚ Inspectors should use the knowledge, skills, and experience called for by their profession to diligently gather evidence and objectively evaluate its sufficiency, competency, and relevancy. Inspectors should seek persuasive evidence and should not presume honesty or dishonesty on the part of those who are providing evidence.
- ✚ The exercise of professional judgment allows inspectors to obtain reasonable assurance that material misstatements or significant inaccuracies in data will likely be detected if they exist.
- ✚ The inspector should carry inspection in manner that external person should not influence the result of inspection

- ✚ Inspectors should use professional judgment in selecting the manner in which inspection is to be performed and the methods that apply to the work, defining the scope of work, selecting the inspection methodology (i.e. written protocols), determining the type and amount of evidence to be gathered and choosing the procedures for their work

### **3.1.4 Checklist prior to inspection**

Pre inspection preparation is a crucial activity that leads to successful inspection. Inspector who wants to inspect bio-fertilizer dealer should plan and make adequate preparations prior to conducting inspection. Planning for inspection enables creation of a conducive environment during inspection. The inspector should ensure that he is equipped with the required inspection items including, but not limited to, the following:

- i) Check list including all items to be verified during the inspection
- ii) Comprehensive list of facilities to inspect
- iii) bio-fertilizer-control documents,
- iv) inspection forms,
- v) sampling tools,
- vi) protective gear,
- vii) Copies of bio-fertilizers regulations and registration guidelines, as well as other applicable regulatory documents, and
- viii) other relevant items

The inspector should ensure that they have the appropriate sampling equipment prior to collection of samples.

The inspector should strive to collect information of bio-fertilizer dealer he is due to inspect.

When the inspection cannot be conducted to all facilities due to whatever reason, facilities to inspect should be randomly selected, unless otherwise advise based on the history of compliance of selected facilities.

### **3.1.5 Conducting inspection**

The inspector should examine documents presented by bio-fertilizer dealers and suppliers with an intention to verify the conformity to the bio-fertilizers regulations and registration requirements using the check-list for inspections. Among factors to consider there is:

- i) history of compliance to the standards
- ii) Registration certificate of the product authorizing documents to conduct bio-fertilizer business by the dealer or the supplier
- iii) Labelling of the products being inspected
- iv) Product storage conditions including code of practice for handling of bio-fertilizer
  
- v) Other relevant information as per the bio-fertilizers regulations

The inspector should carry out visual examination and record the findings in particular with the following: -

- i) Compliance with labelling/markings.
- ii) Packaging requirements
- iii) Storage conditions



#### iv) Nature of the bio-fertilizer

Based on the intention of the inspection, inspector should pick sample in accordance with method of bio-fertilizer sampling. The inspector should properly mark samples at the sampling site after which the relevant inspection forms are filled and signed by both inspector and bio-fertilizer dealer or supplier. A copy should remain with the bio-fertilizer dealer. If the inspector suspects that bio-fertilizer dealer plays foul in terms of bio-fertilizer quality, enforcement actions should be applied including a stop sale order when necessary depending on the type of contravention.

The inspector should send an inspection report to the relevant office of the regulatory office, and at the same time send sample to laboratory for analysis when applicable. Once the inspector laboratory report is received by the inspector, it should be checked for its accuracy, record and communicate to bio-fertilizer dealer with necessary action in regard to the inspection of the product and related batch(es).

### **3.1.6 Types of inspection**

Inspection is conducted to ensure that products in, or entering the marketplaces comply with the (bio)fertilizers regulations and registration guidelines. The main types of inspection include therefore:

- a) Inspection of important material at the port of entry
- b) Inspection of the manufacturing facilities for locally-manufactured bio-fertilizers
- c) Inspection of registration trials
- d) Inspection for marketplace monitoring to ensure that only bio-fertilizers compliant to the (bio)-fertilizers regulations or registration guidelines are legally sold in the marketplace

## **3.2 Identification and Etiquette of the Inspectors**

### **3.2.1 Identification**

An inspector is appointed by the regulatory bodies based on specific terms of relevance. When conducting inspections, inspectors should carry with them the relevant identification document that should be shown to the bio-fertilizers dealers or suppliers before the inspection. The identification document should include at least the name of the regulatory body.

### **3.2.2 Standards of Professional Conduct**

All regulators should observe standards of ethics as per Ghanaian existing code either legally or administratively. Such ethics include those that touch on professional; conduct, attitude, attire, maintenance of good working relationship with industry and public and avoidance of gifts, favours or meals for any official act performed or to be performed.

### **3.2.3 Knowledge Required of a Bio-fertilizer Inspector**

A bio-fertilizer Inspector must have thorough knowledge of the bio-fertiliser requirements and implementing regulations, as well as the other requirements set forth. An inspector must

have certain communication and intuitive skills in order to complete a thorough investigation. The inspector must know how to:

- + Substantiate all statements of witnesses with facts or items of evidence.
- + Collect and document evidence to support a successful civil action, criminal prosecution or seizure.
- + Accurately and clearly write an inspection report, containing all information, data and other records, such as photos, gathered during the inspection.
- + Obtain respect, inspire confidence and maintain the good will of the public, industry and consumers during interviews.
- + Conduct sampling procedures in a safe and professional manner, preserving the chain of custody.
- + Use good interview techniques and detect discrepancies or lack of good faith during interviews.
- + Be accurate, thorough, unbiased and fair while conducting an investigation/inspection and preparing the inspection report.
- + Testify in court.

In addition, an inspector should have adequate knowledge of all the laws and regulations that could be relevant to an inspection in that the facility being inspected may be subject to additional regulations.

### **3.2.4 Confidential Business Information**

When a member of the public, or a representative from a state agency requests access to any information considered confidential, the person handling the request must comply with the procedures set forth. All such requests must be referred to the appropriate office. The written authorization to make copies must contain the following information:

- i) The name of the recipient of the copy.
- ii) The intended purpose for which the copy is to be used.
- iii) The manner in which the copy is to be disposed of after use.

Written guidelines should be put in place and available to the public on request with respect to handling confidential information.

## **3.3 Safety**

### **3.3.1 Introduction**

Manufacturing plants, blenders, processors, elevators, mills and warehouses can be hazardous work environments. Inspection of these places will, at times, include potentially hazardous situations. Personal health and safety must be a top priority for every inspector. To ensure maximum protection under any and all conditions:

- + Prepare and plan for health and safety.
- + Always bring and use safety equipment.
- + Be aware of potential dangers posed at each type of establishment.
- + Exercise care and use common sense at all times.
- + Prevent accidents by specifically requesting and conforming to known safety practices. If there are questions about the safe way to do a job, ask a supervisor for help and instruction.
- + Contact Safety and Health office for pertinent health and safety information and to determine the eligibility and need for medical monitoring.

### 3.3.2 Personal Protective Equipment

Inspections take place in locations that may pose health and safety dangers to inspectors. Additionally, industrial solvents, bio-fertilizers, or bio-fertilizers can corrode or destroy equipment, clothing and footwear. Therefore, it is critical to use Personal Protective Equipment (PPE) during inspections. The following list of PPE is provided as a reference; additional items may be required. Note that all PPE require regular cleaning, visual checks and maintenance.

### 3.3.3 Eye Protection

- Goggles:** Goggles form a tight seal around the eye area and provide an impervious barrier against objects getting into the eyes. They often may be uncomfortable and hard to see through because of condensation. Goggles may be directly vented, indirectly vented or non-vented. Directly vented goggles may be used for particle deflection. They will not, however, be impervious to liquids. Indirectly vented goggles will afford protection from particles as well as most liquid spatters. Non-vented goggles should be used when dealing with anhydrous ammonia.
- Face shield:** Face shields form a plane of protection in front of the eyes. The eyes, nose, and mouth can be protected from direct (perpendicular to the shield) exposure. Objects or substances coming from other directions, however, may get into the face or eyes. When not in use, the face shield may be flipped out of the way to wipe the face. A shield may also be easily used over prescription glasses. A face shield affords only secondary protection and must be used with either goggles or safety glasses.
- Safety glasses** Safety glasses are available in either clear lenses or prescription lenses. Like the shield, they do not afford protection from non-direct exposure. Side guards are available to protect from non-direct exposure and must be used to make them more effective. If glare may be a problem, tinting may be added.

### 3.3.4 Foot Protection

Types of foot protection include:

- Footwear (shoes or work boots):** Footwear should be comfortable to wear, fit properly and provide proper support. Secondly, it must have a steel toe or protective support in case objects are dropped on the foot. The sole and heel of the footwear must be appropriate for the working environment. Do not wear slick-soled shoes or sneakers. Use proper protective mechanisms to prevent your feet from being exposed to bio-fertilizers. Workers have become seriously ill from exposure through shoes or boots due to the footwear material soaking up the pesticide and exposing the person through skin contact. A good practice is to “waterproof” footwear; this function should be performed regularly.
- Rubber/neoprene boots/galoshes:** Ordinary work boots or shoes are not suitable when handling bio-fertilizers. Rubber boots or neoprene boots must be worn. Rubber boots provide a protective barrier against water and some solvents. They also may be easily cleaned. Neoprene boots will afford

protection from various chemicals and solvents. Either “gum” boots, pull-over boots, or buckle boots may be used.

**Tyvek booties:** Tyvek booties can be worn over shoes/boots and add a large degree of protection from absorption of chemicals into shoes, boots, and the body.

### 3.3.5 Hand Protection

Hand protection comes in many forms. The class and material of gloves used must be taken into account when selecting the proper type for use. Certain gloves may have long shanks and afford protection for the wrist or lower arm. Be aware that cuffs present a hazard by allowing material to collect in the cuff or getting caught in machinery. When necessary, inspectors must use the following to protect their hands:

**Work gloves (cotton or jersey):** Cotton or jersey work gloves will provide warmth in the winter and some protection from dirt and blisters. Be careful not to use cotton or cloth gloves when working with bio-fertilizers, chemicals, or solvents. Cloth gloves will act as a wick and absorb these products, allowing continued contact with the skin.

**Disposable latex gloves:** Disposable latex gloves are easy to use and afford protection from some bio-fertilizers, seed treatments, dirt, grease, non-corrosive materials or liquids. They are easy to use and readily disposable. However, some people may be allergic to latex. If a rash occurs, seek proper medical attention. A disadvantage of latex gloves is that they do not afford protection against corrosive materials or certain chemicals. When using latex gloves, determine for what products the gloves are rated.

**Heavy rubber gloves:** Heavy rubber gloves afford the best protection when working around bio-fertilizers or other similar products. These gloves are easily rinsed or cleaned when liquids are spilled on them.

### 3.3.6 Ear Protection

Types of ear protection include:

**Disposable foam plugs:** Disposable foam plugs are inexpensive, easily stored and used; but they may be uncomfortable to wear. Make sure the plugs are rated with the proper protection for the environment you will be working in.

**Padded hearing protectors:** Padded hearing protectors are the typical “headphone” type protectors. They afford noise protection as well as a cover for the ear. Usually they are rated higher than typical plugs and prevent compacting in the ear canal.

### 3.3.7 Nasal, Mouth and Respiratory Protection

Respiratory protection equipment includes:

**Disposable dust masks:** Disposable dust masks are easily worn and afford little or no protection from bio-fertilizers. However, they may be used for general dust and limited airborne particulates. Dust masks only prevent

particles from passing through, and offer little or no protection against caustic or dangerous fumes, bio-fertilizers, etc.

***Respirator:***

Respirators usually contain various filters that can be interchanged depending upon the hazards of the working environment. Filters are rated for particular substances. Be aware of the respirator's rating and the protection it affords. Likewise, be aware of the respirator's limitations. Respirators must never be used in oxygen-deficient environments. NOTE: Facial hair may prevent a tight seal.

- ✚ Test according to the manufacturer's directions to ensure an air-tight seal.

- ✚ Achieving an air-tight seal may present a greater problem for women than for men because many respirators are sized to fit a man's face. Ensure that a tight fit can be achieved prior to leaving the office.

***Air packs:***

Self-contained breathing apparatuses (SCBA) contain a mask, oxygen tank, and regulator. Before using SCBA, inspectors must have attended the 40-hour Hazard Materials Training course and obtained a physician's approval to wear it. It is extremely important that two SCBA-trained people be on hand when entering oxygen deficient environments.

### **3.3.8 Head Protection**

Proper head protection is recommended during most inspections. Steel or moulded hats protect the head and skull from falling objects or head height obstacles.

### **3.3.9 Back Protection**

When lifting heavy objects, use a proper back support or lift belt.

### **3.3.10 Clothing Protection**

The inspector should wear clothing protection to prevent-cross contamination of sites and/or samples as well as providing protection to the inspector and preventing contamination of the inspector's home or office. Inspectors should use the following:

***Cloth coveralls:*** Protective outerwear is available to be worn over your normal clothing. Lightweight coveralls can be used during the summer months while insulated coveralls can be used in the winter. These coveralls will keep clothing from receiving stains while in dirty work environments.

***Tyvek coveralls:*** Tyvek is an extremely lightweight, disposable paper-like substance that is extremely hard to tear. Tyvek is the preferred material because, unlike cloth, it does not absorb and, if contaminated, can be discarded. Tyvek is chemical-resistant.

### **3.3.11 General Safety Precautions**

Inspectors must inform facility personnel where they are going to be within the facility in the briefing meeting preceding the inspections. Inspectors should specifically request information on possible dangers or areas to avoid during the inspection during the discussion.

Communication prior to the inspection is generally not recommended, except for special cases that will be defined in the inspection protocols. Follow all safety requirements established by facility. For example, vehicular traffic within the plant grounds may not follow normal movement patterns nor obey usual traffic rules. Also, the nature and size of equipment used may make it difficult for the driver to see persons working nearby.

- + Wearing jewellery, ties, loose flowing clothing, having long flowing hair, etc. can pose a safety hazard to the inspector around equipment or machinery.
- + Prior to entering the plant or facility, inquire about the firm's safety policies. Many firms require visitors to wear a hard hat and/or safety glasses. Hardhats should be worn at all times while on manufacturing and warehouse premises.
- + Safety shoes with non-slip soles and heels should be worn. Clothing should be close fitting. Make sure laces are tied.
- + Flashlights should be carried, especially when work assignments involve the upper floors or basements.
- + Dust masks or properly rated respirators should be worn in dust-laden environments.
- + If there is a need to use a protective device that has not been supplied or if there are safety concerns, notify a supervisor.
- + While in the establishment, make sure equipment is secure during transit. A pen in a shirt pocket may fall out during the inspection and may fall into the firm's equipment or product. A probe, flashlight, or folder may slip or fall during climbing or conducting the inspection. This may cause personal injury or injury to other employees.

### **3.3.12 Awareness**

An inspector must not enter a bio-fertilizer manufacturing/processing/packaging plant/warehouse, unless accompanied by the facility's supervisory personnel. Bio-fertilizers dealer/supplier should be formally informed that is a severe contravention to deny assistance, or hide information or products considered for the inspections. The dealer/supplier shall offer maximum cooperation to the inspector. Hence, prior to the inspection of the facility a meeting with the dealer/supplier or his representative shall be held prior to inspecting the facility and products.

Once in the plant, inspector should endeavour to know the location of exits, telephones, and first aid equipment, and especially emergency evacuation routes and procedures. One should read and follow all warning signs.

When entering an elevator, mill, or warehouse from bright outside light, vision may be temporarily impaired. Stop and let your eyes adjust before continuing the inspection. Be aware of the surroundings. Avoid stepping on manhole covers since they may slide from underfoot.

Be conscious of the machinery being used in the vicinity. Observe conditions surrounding the various products to be sampled, with emphasis on the danger of front-end loaders, hopper and tank cars, forklifts, conveyor belts, motor drives, mixers or blenders, welding and cutting equipment, drag and screw conveyors, falls from heights and electrical equipment. Stay clear of machinery, whether it is operating or not. The "dead" machinery may be started by a remote control switch located in another part of the plant. Do not sit or step on a motionless conveyor belt. Cross over conveyors only on cross bridges or walk around the belt end.

Watch for wet floors. Dust caused by loading or unloading bio-fertilizer, or related products can mix with the moisture on the floors, making them extremely slippery and hazardous. When the air is dust laden, the ability to see is reduced. This can be dangerous. In this environment, protect both eyes and respiratory system with the proper equipment.

High pressure air lines must not be used to blow dust from clothing or the body. Foreign matter such as metal fragments, oil, or water can be blown under the skin or into eyes, causing a painful or serious injury. There should be NO SMOKING at any time in an elevator, mill, or facility.

### **3.3.13 Machine and Equipment Safety**

When working around machinery, always take the following precautions:

Never attempt to operate any machinery.

Never remove a machinery guard or shield on a piece of equipment while it is running. Guards must never be removed unless absolutely necessary for equipment inspection. If guards are removed, steps need to be taken to ensure the equipment is not started and the guards are replaced promptly once the inspection is complete. Some agencies do not permit removal under any condition. If an emergency arises, be prepared by discussing the topic with a supervisor.

### **3.3.14 Safety Signs**

Respect all safety signs in the plant; they are posted for everyone's safety. Failure to obey signs can cause injury. The words "caution" and "warning" are there for a reason. Pay attention to directions provided.

### **3.3.15 Electrical Safety**

Never tamper with electrical equipment. Electricity can kill quickly.

### **3.3.16 Confined Spaces**

Confined space entry has resulted in more deaths and injury than any other source in the industry, therefore, it is essential to recognize and carefully evaluate the situation prior to entry.

### **3.3.17 Safety While Sampling**

Prior to sampling, assess the conditions to be encountered when inspecting trucks, rail cars or storage areas. Be cognizant of fumigant warning-agent odours; however, remember that some toxic fumigants have no odour. Rather than take any chances, check with management to eliminate any risk of exposure. DOT regulations and pesticide labels usually require that warning signs be placed on rail cars containing fumigated commodities. If there is a fumigant notice on the car, especially if it has a recent date (3 days or less), or if you detect a fumigant odours, do not open it. Notify the firm management to have a qualified person determine if it is safe to open the car. The firm's qualified person must open the doors on both sides of the

car and allow the car to air out for a prescribed length of time before allowing anyone to enter. Remember, some fumigants may not have detectable odours, but are still a hazard.

Do not enter trucks, rail cars, or storage areas during the application of these materials, or enter where the materials have been applied unless the atmosphere has been certified safe by a competent person.

### **3.4 Powers of Bio-fertilizer Inspector**

Bio-fertilizer inspector may, at any reasonable time:

- ✚ examine any place, premises, vessel or vehicle in respect of which he/she has reason to believe that, on or in it there is manufactured, processed, prepared, graded, classified, packed, marked, labelled, held, bottled, removed, transported, exhibited, sold or used any bio-fertilizer,
- ✚ examine all books and documents at any place, premises or vehicle in respect of which he has reasonable grounds for believing that they relate to any and make copies of or extracts from such books or documents,
- ✚ examine any operations or processes carried out at any place or premises in connection with manufacturing, processing, treatment, preparation, grading, classification, packing, marking, labelling, holding, bottling, removal, transport, exhibition, selling or use of any bio-fertilizer,
- ✚ demand from the owner or any person having the custody of any book or document in connection with bio-fertilizer business and demand explanation to any record or entry therein
- ✚ seize any book, document, bio-fertilizer which may furnish as a proof of an offence to provisions of the Ghanaian bio-fertilizer Act/policy/regulations,
- ✚ may issue stop sale order until he/she is clear of the doubt, including sampling and analysis of bio-fertilizer,
- ✚ sample or cause sampling of bio-fertilizer from premise, vessel, vehicle, processing, exhibition or any other place as he may wish

### **3.5 Responsibilities of Bio-fertilizer Inspector**

Any bio-fertilizer inspector should be responsible for:

- ✚ Processing registration or cancellation of bio-fertilizer dealer and sterilizing plant for manufacturing of bio-fertilizer
- ✚ Conducting inspection of bio-fertilizer or sterilizing plant
- ✚ Keeping information and records of:
  - a) Every bio-fertilizer dealer and his/her registration in their area of jurisdiction,
  - b) Type and quantity of bio-fertilizer,
  - c) Source of bio-fertilizer
  - d) Quantity sold and quantity offered for sale in their area of jurisdiction,
  - e) Bio-fertilizer export or transferred from the area
  - f) Any other information regarding bio-fertilizer business necessary for purposes of quality control.

### **3.6 Reports**

Information gathered and presented by inspectors is essential to the success of the enforcement program. The inspector must be able to prepare clear, objective and well-



documented written inspection reports. The main purpose of a written inspection report is to clearly and concisely communicate a complete and factual record of the inspection process, observations and results to the reader. To communicate effectively, the report must be a complete and accurate record of what was discovered and what occurred during the inspection. The report should not contain any opinions of the inspector and should not make any conclusions of law.

Immediately after completion of bio-fertilizer inspection mission, the inspector should write a narrative inspection report as soon as possible. At a minimum, an inspection report for a suspected misuse or violation should include reference to all information gathered. The report should be detailed enough for instance, all relevant information such as explanation of samples, photographs and other evidence collected; and a description of any follow-up that may be warranted. Attach all completed forms and documents gathered during the course of the inspection to the narrative report as exhibits.

### 3.6.1 Steps in Writing Reports

**Planning:** By planning how facts must be presented in the inspection report prior to the inspection, the inspector can improve the quality of the inspection report as well as the inspection itself. It should be noted that some elements that will need to be included in or with the report (i.e., samples analysis) may not be available at the time of report preparation. Do not delay preparing the report while waiting for sample analysis. Sample analysis can be added as an exhibit to the report when it becomes available.

**Organize Material:** All information gathered during the inspection must be reviewed for relevance and completeness. This includes inspection report forms, field notebooks and checklists. The field notebook and/or inspection checklists are useful tools for developing the narrative report, but cannot replace a narrative report. Any identified gaps in the information must be resolved by follow-up telephone calls or follow-up inspections. The material must then be organized in the order that it will be presented in the report.

**Writing:** While writing the inspection report, the following should be kept in mind:

- ✚ The inspection report should be a complete record of what occurred at the inspection, including conversations that took place, documentation that was collected and physical samples that were collected. Photographic evidence should be included when appropriate.

- ✚ Only the facts as to what was observed should be reported. Do not include opinions or conclusions of law. Keep the reader in mind. When preparing an inspection report, assume that the reader knows nothing about the case except what is in the report. The report must construct a complete and accurate picture of the entire inspection, step-by-step.

- ✚ The report should be peer-reviewed before it is finalized.

**Evaluate:** After writing the report, it should be reviewed from the viewpoint of the reader and the following questions answered:

- ✚ Does it answer the questions—who, what, when, where, why and how?
- ✚ Is each asserted fact supported, with a citation provided, by a document, picture, sample, recorded observation or statement from an individual?
- ✚ Is it fair, concise, complete, accurate and logical? Is any part ambiguous?
- ✚ Does it communicate clearly?
- ✚ Is there any other information needed to fulfill the purpose of the investigation?
- ✚ Can supervisors and reviewers make appropriate decisions based on this report?
- ✚ Are any further inquiries necessary?

Proofread the report to check for inconsistencies, unnecessary repetition, tone, omissions and typographical errors.

**Rewrite:** Correct those portions of the narrative that were identified as needing improvement.

Essentially, a good report should be: fair; accurate, evidence based, clear, logically presented and concise.

### 3.7 Warehouse for Storage of bio-fertilizers

Inspectors should ensure that:

- ✚ Bio-fertilizer are kept in a way to ensure compliance of its quality;
- ✚ the warehouse for storage is located in a clean and tidy site with arrangements provided for destruction and disposal of waste and away from areas liable to flooding and away from drinking water sources;
- ✚ the site is free from rodent activity and should have a good drainage system;
- ✚ the construction of the warehouse is climatically suitable and damp-proof with structurally sound walls and roof that effectively keeps out rain, provides ventilation to allow fumes and heat to escape in case of fire and at the same time provide protection against direct sunlight;
- ✚ the store doors are sound, well-fitting and secure and the windows and ventilators are in good repair and screened to prevent access by birds or rodents;
- ✚ the internal walls of warehouse are structurally sound, non-flammable type and with all piping and electrical wiring sealed;
- ✚ floors are made of concrete with a load bearing capacity sufficient to withstand the weight of the stock, racking and any mechanical handling equipment to be used. They should also be impervious to liquids, free from cracks and facilitate cleaning;
- ✚ the building is designed so as to enable escape in case of emergency from any enclosed area and that emergency exits are clearly marked;
- ✚ it permits reasonable movement of materials and enough space to allow hygienic working conditions and clear access to fire-fighting equipment.

### 3.8 Significance and importance of dealing in registered bio-fertilizer

Given the nature of bio-fertilizer and their role in agricultural production systems, the inspectors should be keen in ensuring that circulation of bio-fertilizer products for research or commercial purposes can be allowed after it has been proved to be safe, efficacious, and effective in consistent with label claims, and must be registered or certified for use in the

country. This is based on the fact that, materials such as bio-fertilizers which contain soil microbes, although it is used to sustain good soil condition, are treated as environmental issues.

### **3.9 Inspection Obligations of a Bio-fertilizer Dealer**

Bio-fertilizer inspector should endeavour to ensure that every bio-fertilizer dealer is aware of their obligations. That, every bio-fertilizer dealer:

- + is responsible for the quality of any bio-fertilizer she/he sells or offers for sale,
- + keeps bio-fertilizer in a way to ensure compliance of its quality as per code of practice for storage and transport of the bio-fertilizer,
- + gives an inspector, reasonable assistance to enable the inspector to carry out his/her duties and functions according to the relevant bio-fertilizer regulatory policy,
- + arranges the packages in such a way to enable the Inspector to reach all packages and draw samples.
- + does not move any product detained by the Inspector unless with a written consent of the Inspector indicating that the seized product should be placed in a safer location.
- + can appoint an agent with knowledge, ability and appropriate facilities to maintain the quality of the bio-fertilizer offered for sale. An agent so appointed should have a valid registration certificate for dealing with bio-fertilizer business issued by relevant regulatory authority.
- + respects the decision made by the Inspector as when he issues stop sale order on grounds that any bio-fertilizer lot is being sold without having reached minimum prescribed standards or in violation of any provisions of the Act and bio-fertilizer Regulations.
- + not offer for sale products under stop sale order unless the inspector is satisfied that all conditions for release as provided for in the relevant bio-fertilizer Act/legislation/regulatory policy have been complied with, and importantly confirms it in writing.
- + keeps information and records regarding his bio-fertilizer business.
- + should keep information and records of each bio-fertilizer in regards to:
  - i) Type of bio-fertilizer in their custody,
  - ii) Source,
  - iii) Date and quantity sold and quantity offered for sale;
  - iv) batch number,
  - v) Any other information regarding bio-fertilizer business necessary for purposes of quality control

### **4 Bio-fertilizers regulations and registration guidelines**

Inspectors should have adequate understanding of the bio-fertilizers regulatory environment including bio-fertilizers policy, act, regulations, and registration guidelines. In general, bio-fertilizers are defined in the fertilizers policy, act, and regulations. Given their particular nature, bio-fertilizers specific registration guidelines are necessary as they contain living microorganisms contrary to inorganic and organic fertilizers products. In this chapter, the biofertilizers manual will be used only for the training purposes and the registration guidelines for bio-fertilizers are an integral part of this training manual. Bio-fertilizers dealers and suppliers should be adequately informed about the registration guidelines; the documents

should be available to them on request. The same applies to the standard operational procedures (SOPs) for quality control.

The registration guidelines have been developed by the regulatory officers. Inspectors should therefore contact the regulatory body if they need clarification on particular provisions of the guidelines.

## 5 Labelling requirements

The registration guidelines also include labelling requirements. However, it is important to elaborate more about those guidelines in this chapter as most of the inspection decisions will be based on the labelling requirements in a way or another.

### 5.1 Product label

Based on the performance trial data, the regulatory officer should be able to ensure that the label is amended accordingly into a commercial label. A copy of the approved commercial label should thereafter be circulated to all inspectors in the regions in the country where the sale of the product is expected. The commercial label should include a registration number granted by the relevant regulatory body as a seal of approval. Equally important, the label of a bio-fertilizer sold in Ghana should be at least in both official and national languages.

### 5.2 Specific requirements for labelling and packaging

Bio-fertilizer inspector should be conversant with the labelling requirements. The labelling requirements are categorized into two; (i) *mandatory*: the items that must appear on the label; and (ii) *optional*: important items but not obligatory that it should appear on the label. Detailed labelling requirements are shown in Table 1 in addition to the information in the registration guidelines.

Table 1. Labelling requirements for bio-fertilizers

Item	Mandatory	Optional	Remarks
Product name	✓		
Brand name		✓	
Net weight/volume (in standard (SI) units)	✓		
Lot/Batch number	✓		
Registration number	✓		
Full Company contact details: •	✓		contact address including the physical address of the place of manufacture, Telephone/email/Fax

Manufacture Date	✓		
Expiry date	✓		
Direction for use: <ul style="list-style-type: none"> <li>• Should have understandable Units of measure (SI units) and application rates;</li> <li>• Pre-planting treatment period</li> </ul>	✓		Specifies application rates, frequency of application, target crops, application equipment, and method of application, among others
Guarantee analysis/Microbial Density	✓		A statement that shows the content (concentration of the active ingredients) of the product, i.e. CFU of active ingredient(s) per gram of finished bio-fertilizer product
Sellers warranty/guarantee statement		✓	In case of damage or product failure when product is used as per the direction for use
Cautionary statement	✓		
Label claims		✓	
List of active ingredients		✓	
Website		✓	
Storage, Handling	✓		for both the biofertilizer and containers
Disposal	✓		
Barcode		✓	Propose format of label
Net weight	✓		Net weight in local equivalence of the SI units of measure (optional)

### 5.3 Other requirements

Besides the detailed requirements in Table 1, the inspectors ensure that:

- the labels is written in the official language of Ghana,
- in the case of imported products, manufacturers information should be mandatory on the label,
- Planting window or pre-planting treatment period (when applicable) is indicated,
- if an importer changes the source/manufacturer of a product, then the new product must be subjected to complete regulatory process just like a new product; unless the former manufacturer confirms that the products are identical for both the active ingredients and the carrier material as well as the quality assurance and control of the new manufacturer (depending on the arrangement between the two manufacturers).

- Product labels should not have any incorrect or misleading information, mark, brand, or name that would tend to deceive or mislead the end-user with respect to the composition or benefits of using the product.
- Ensure that the approved product label is the only commercial label found in the marketplace for the registered bio-fertilizer.
- All information on the labels is printed conspicuously, legibly (font size that would be legible from a normal distance without the aid of magnifying devices) and indelibly.
- Ensure that, if the product is packaged outside the country of interest, and contains the country's address on the label, and is imported for resale, the words "imported by" or "imported for" precede the country's address, in addition to the geographic origin of the pre-packaged product as required above.

## 6 Sampling and handling of products

Sampling is one of the most important functions of the analytical process. No matter how accurately the final laboratory analysis or inspection is performed, the results obtained cannot be any more accurate than the degree of accuracy with which the initial sampling operation was performed. The basic purpose of sampling is to collect a

manageable mass of material that is representative of the total mass of material from which it was collected. The manageable mass of material is called “sample” and is subject to certain preparation procedures, which render it suitable for either physical or chemical analysis. Given the core mandate of bio-fertilizer inspectors, they have to be competent in sampling procedures to ensure accurate quality evaluation of bio-fertilizer products. The manner by which increments are collected or how sampling is carried out is probably the most important operation in inspection. Since it is required to collect increments from all parts of the lot, it is necessary that the total lot be accessible. In other words, it is of fundamental importance that all particles in the lot have the same probability of being included in the final sample. This is one of the key rules of sampling.

Rule of thumb: A bio-fertilizer sample should be only taken by a qualified inspector to take such samples containing living microorganisms. The samples should be taken in the presence of the dealer/supplier or their authorized representative. The dealer/supplier should sign on the sampling form.

Bio-fertilizer sampling is one of the most important aspects of bio-fertilizer quality control. Improper sampling not only damages the ethics of quality control system but also discourages honest bio-fertilizer dealers from maintaining quality.

Details related to the sampling requirements, principles, drawing samples, and scale of sampling is outlined in the registration guidelines. A sample form is important to ensure that the important information about the sample and sampling conditions are properly documented and confirmed by the dealer/supplier or his/her authorized representative.

## 7 References

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## Annex 9: Selected molecular techniques

### 1 DNA and DNA Extraction

DNA stands for Deoxyribo Nucleic Acid. It is located in the nuclei of cells, which make up the body. Consequently, DNA can be considered as one of the building blocks of the body.

To understand the exact structure and function of the body it is essential to know what a cell is and how it is structured.

#### 1.1 The Cell

The cell is the basic structure of the body. The human body for example, is built of billions and trillions of cells. Cells of different organs vary according to their function.

Each cell contains the hereditary material and can make copies of themselves by reproducing and multiplying except the nerve and brain cells. After a specific life span the old cells die off.

Parts of the cell are called organelles. Human cells contain the following major parts:

- Nucleus – This is central part of the cell that carries the blue print for the cell functioning and tells the cell when to grow, reproduce and die. It also houses DNA.
- Mitochondria – These are the powerhouses of the cell and produce energy for the various activities of the cell.
- Cytoplasm – This is a jelly like fluid within the cell in which the other organelles float.
- Endoplasmic reticulum (ER) – This helps in processing the molecules (e.g. proteins) created by the cell.
- Ribosomes – These lie over the ER and process the genetic instructions or the blueprints within the DNA to produce new proteins. These can also float freely in the cytoplasm.
- Lysosomes and peroxisomes – These help in digesting foreign bacteria that invade the cell, rid the cell of toxic substances
- Cell membrane – This is the outer lining of the cell.

#### 1.2 The Chromosomes

Within the nucleus the DNA strands are tightly packed to form chromosomes. During the cell division the chromosomes are visible.

Each chromosome has a constriction point called the centromere from where two arms are formed. The short arm of the chromosome is labeled the “p arm.” The long arm of the chromosome is labeled the “q arm.”

Each pair of chromosome is shaped differently by the location of the centromere and the size of the p and q arms.

Humans normally have 23 pairs of chromosomes, for a total of 46. Twenty-two of these pairs, called autosomes, look the same in both males and females.

The 23rd pair is called the sex chromosomes and differs between males and females. Females have two copies of the X chromosome or XX, while males have one X and one Y chromosome.

#### 1.3 The Genes

Genes are hereditary material that lies within the cell nucleus. Genes, which are made up of DNA, act as instructions to make molecules called proteins.

The Human Genome Project has estimated that human genome has 20,000 to 25,000 genes. Every person has two copies of each gene, one inherited from each parent. These are mostly similar in all people but a small number of genes (less than 1 percent of the total) are slightly different between people and this forms the basis of paternity tests and DNA analysis.

#### 1.4 Where is DNA found?

The DNA is the hereditary material that lies within the nucleus of all cells in humans and other living organisms. Most of the DNA is placed within the nucleus and is called nuclear DNA. However, a small portion of DNA can also be found in the mitochondria and is called mitochondrial DNA or mtDNA.

#### 1.5 What is DNA made of?

DNA contains four chemical bases known as nucleotides:

- Adenine (A)
- Guanine (G)
- Cytosine (C)
- Thymine (T)

Each of these nucleotide consists of a base, sugar and a phosphate molecule.

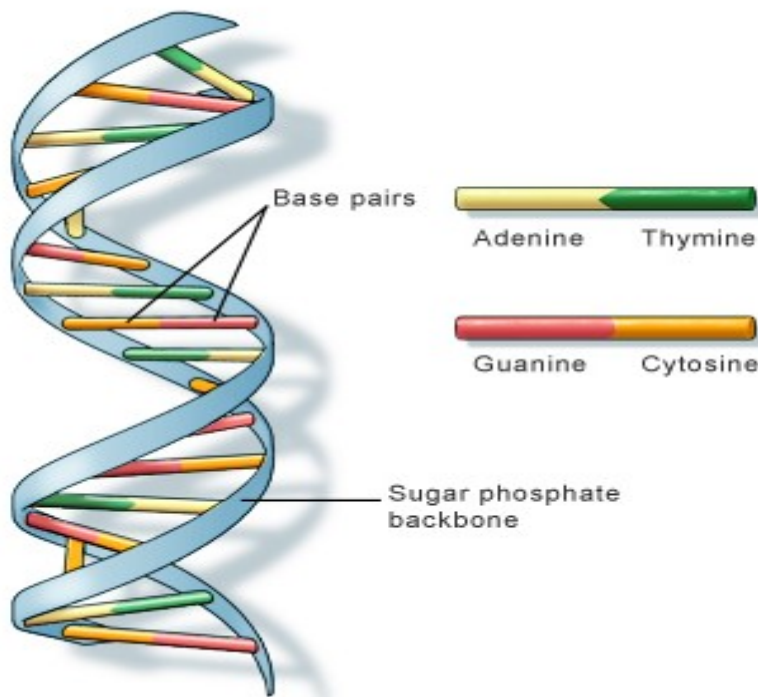


Figure (?) title? and acknowledge the source?

#### 1.6 DNA base pairs

DNA bases pair up with each other, A with T and C with G, through hydrogen-bond to form units called base pairs. Each base is also attached to a sugar molecule and a phosphate molecule. DNA in humans contains around 3 billion bases and these are similar in two persons for about 99% of the total bases.

These bases are sequenced differently for different information that needs to be transmitted. This is similar to the way that different sequences of letters form words and sequences of words form sentences.

### 1.7 Nucleotides and the double helix

A base, sugar, and phosphate in combination forms a nucleotide. Nucleotides are arranged in two long strands that form a spiral called a double helix. This looks like a twisted ladder and the base pairs form the rungs of the ladder and the sugar and phosphate molecules form the sides of the ladder.

### 1.8 How does DNA replicate itself?

The DNA can make copies of itself. Both the strands of the DNA open up and make a copy of each and become two DNA stands. Thus each new DNA has one copy of the old DNA from where the copy is made.

### 1.9 Mitochondrial DNA

The mitochondria contain small amount of DNA. This genetic material is known as mitochondrial DNA or mtDNA. Each cell contains hundreds to thousands of mitochondria that lie within the cytoplasm. Mitochondrial DNA contains 37 genes that help it to function normally. Thirteen of these genes provide instructions for making enzymes involved in energy production by oxidative phosphorylation. The rest of the genes help in making molecules called transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) that help in protein synthesis.

Deoxyribonucleic acid (DNA) molecules are informational molecules encoding the genetic instructions used in the development and functioning of all known living organisms and many viruses. Along with Ribonucleic Acid, RNA, and proteins, DNA is one of the three major macromolecules that are essential for all known forms of life. Genetic information is encoded as a sequence of nucleotides, recorded using the letters G, A, T, and C. Most DNA molecules are double-stranded helices, consisting of two long polymers of the simple units, nucleotides, with backbones made of alternating sugars (deoxyribose) and phosphate groups, with the nucleobases (G, A, T, C) attached to the sugars. DNA is well-suited for biological information storage, since the DNA backbone is resistant to cleavage and the double-stranded structure provides the molecule with a built-in duplicate of the encoded information.

Within cells, DNA is organized into long structures called chromosomes. During cell division these chromosomes are duplicated in the process of DNA replication, providing each cell its own complete set of chromosomes. Eukaryotic organisms (animals, plants, fungi, and protists) store most of their DNA inside the cell nucleus and some of their DNA in organelles, such as mitochondria or chloroplasts. Prokaryotes (bacteria and archaea) store their DNA only in the cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA. These compact structures guide the interactions between DNA and other proteins, helping control which parts of the DNA are transcribed.

DNA Extraction is simply the removal of deoxyribonucleic acid (DNA) from the cells or viruses in which it normally resides.

### 1.10 What is it used for?

Extraction of DNA is often an early step in many diagnostic processes used to detect bacteria and viruses in the environment as well as diagnosing disease and genetic disorders. These techniques include but are not limited to -

- Fluorescence In Situ Hybridization (FISH): FISH is a molecular technique that is used, among other things, to identify and enumerate specific bacterial groups.
- Terminal Restriction Fragment Length Polymorphism (T-RFLP): T-RFLP is used to identify, characterize, and quantify spatial and temporal patterns in marine bacterioplankton communities.
- Sequencing: Portions of, or whole genomes may be sequenced as well as extra chromosomal elements for comparison with existing sequence in the public data base.

### 1.11 How does it work?

Outline of a basic DNA Extraction -

1. Break open (lyse) the cells or virus containing the DNA of interest- This is often done by sonication or bead beating the sample. Vortexing with phenol (sometimes heated) is often effective for breaking down protienacious cellular walls or viral capsids. The addition of a detergent such as Sodium dodecyl sulfate (SDS) is often necessary to remove lipid membranes.
2. DNA associated proteins, as well as other cellular proteins, may be degraded with the addition of a protease. Precipitation of the protein is aided by the addition of a salt such as ammonium or sodium acetate. When the sample is vortexed with phenol-chloroform and centrifuged the proteins will remain in the organic phase and can be drawn off carefully. The DNA will be found at the interface between the two phases.
3. DNA is precipitated by mixing with cold ethanol or isopropanol and then centrifuging. The DNA is insoluble in the alcohol and will come out of solution, and the alcohol serves as a wash to remove the salt previously added.
4. Wash the resultant DNA pellet with cold alcohol again and centrifuge for retrieval of the pellet.
5. After pouring the alcohol off, the pellet is set to dry, the DNA can be re-suspended in a buffer such as Tris or TE.
6. Presence of DNA can be confirmed by electrophoresing on an agarose gel containing Ethidium bromide, or another fluorescent dye that reacts with the DNA, and checking under UV light.

### 1.12 Ribosomal RNA

To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. Examples of genes in this category are those that define the ribosomal RNAs (rRNAs). In Bacteria, Archaea, Mitochondria, and Chloroplasts, the small ribosomal subunit contains the 16S rRNA (where the S in 16S represents Svedberg units). The large ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA. Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon (A group of distinct genes that are expressed and regulated as a unit). There may be one or more copies of the operon dispersed in the genome (for example, E coli has seven). The Archaea contains either a single rDNA operon or multiple copies of the operon.

Ribosomal RNAs in Prokaryotes:

Name	Size (nucleotides)	Location
5S	120	Large subunit of ribosome
16S	1500	Small subunit of ribosome
23S	2900	Large subunit of ribosome

The 16s rDNA sequence has **hyper variable regions**, where sequences have diverged over evolutionary time. **Strongly conserved** regions often flank these hyper variable regions. Primers are designed to bind to conserved regions and amplify variable regions.

## 2 Polymerase Chain Reaction (PCR)

Sometimes called "molecular photocopying," the polymerase chain reaction (PCR) is a fast and inexpensive technique used to "amplify" - copy - small segments of DNA. Because significant amounts of a sample of DNA are necessary for molecular and genetic analyses, studies of isolated pieces of DNA are nearly impossible without PCR amplification. Often heralded as one of the most important scientific advances in molecular biology, PCR revolutionized the study of DNA to such an extent that its creator, Kary B. Mullis, was awarded the Nobel Prize for Chemistry in 1993. In order to use PCR, one must already know the exact sequences which flank (lie on either side of) both ends of a given region of interest in DNA (may be a gene or any sequence).

### 2.1 What is it used for?

Once amplified, the DNA produced by PCR can be used in many different laboratory procedures such as

- Mapping Human Genome .
- Laboratory and clinical techniques such as DNA fingerprinting.
- Diagnosing disease and genetic disorders.
- Detection of bacteria and viruses in the environment.
- Analysis of microbial communities.

### 2.2 DNA purification

Many procedures in molecular biology require an initial pure sample of DNA. These procedures include PCR, sequencing, gene cloning, blotting, and DNA profiling. Purification of DNA involves removing it and other constituents from the cell, separating it from various other cell constituents, and protecting it from degradation by cellular enzymes. Isolation procedures must also be gentle enough that the long DNA strands are not sheared by mechanical stress.

DNA can be isolated from almost any cellular source. White blood cells and cheek cells taken directly from humans are most commonly used for diagnostic purposes, but skin, hair follicles, semen, and other tissues can be used for forensic analysis. Cells grown in Petri dishes or in suspension can also be used. The cells are isolated from any surrounding fluid (such as blood serum) by centrifuging them—spinning them at high speeds—and then are re-suspended in a buffer solution. The buffer prevents rapid or dramatic changes in pH, which can interfere with subsequent reactions. To break open the cell membranes, a detergent is added to the buffer. Sodium dodecyl sulfate (SDS) is often used for this purpose. The detergent also helps remove proteins and lipids in the cell.

The buffer also contains ethylenediaminetetraacetic acid (EDTA), which is a chelator. Chelators are molecules that act as scavengers for metal ions in solution. They take metals by completely engulfing them . This is important because DNase, an enzyme that digests DNA, is present in the cell and would destroy the long DNA strands if it was active. DNase activity requires magnesium ions, and EDTA removes them from solution, preventing DNase from cutting up the DNA. RNase is also present in the buffer at this step, to break up the RNA present in the cells.

The solution is then treated with proteinase K, a highly effective enzyme that inactivates all types of proteins. This enzyme can also be used earlier in the procedure to break apart clumped cells. Unlike many proteins, proteinase K remains active at elevated temperatures, so the solution can be heated to about 55

°C to aid protein inactivation and removal by the detergent. This step may last between two and sixteen hours.

Once the cells are broken open and the RNA, proteins, and lipids have been dissolved in the buffer, the DNA must be separated from these materials. One standard technique uses phenol to remove the proteins, leaving DNA and other water-soluble materials behind. The DNA is then extracted from the water phase using chloroform and precipitated from the chloroform using ethyl alcohol mixed with sodium acetate salt. The DNA is then removed either by spooling the long threads onto a glass rod, or by spinning it out of solution using a centrifuge. The DNA is then re-suspended in buffer.

Another technique for separating the DNA from the mixture avoids the use of phenol and chloroform, which are toxic. Instead, the proteins are "salted out" by adding a concentrated salt solution and then removed by centrifuging. In another method, DNA is adsorbed onto very small glass beads, in the presence of "chaotropic" salts, which disrupt protein structure. The beads are removed (or washed in place), and the DNA is released by changing the salt concentration.

Once a pure sample of DNA has been obtained, the fragments it contains may be separated on the basis of size by gel electrophoresis. Specific sequences can be identified by Southern blotting using probes with complementary sequences, and they can then be cut out of the gel for further use, such as cloning. If the sample is small, the DNA can be amplified by PCR..

### **2.3 PCR in practice**

PCR is used to amplify a short, well defined part of a DNA strand. The PCR provides an extremely sensitive means of amplifying small quantities of DNA. The PCR process can copy only short DNA fragments, usually up to 10 kb.

PCR, as currently practiced, requires several basic components which include:

- DNA template, which contains the region of the DNA fragment to be amplified
- Two primers, which determine the beginning and the end of the region to be amplified
- Taq polymerase, which copies the region to be amplified
- Nucleotides, from which the DNA polymerase builds the new DNA
- Buffer, which provides a suitable chemical environment for the DNA polymerase

The PCR reaction is carried in a thermal cycler, a machine that heats and cools the reaction tubes within it to precise temperature required for each step of reaction. To prevent evaporation of the reaction mixture, a heated lid is placed on top of the reaction tubes or a layer of oil is put on the surface of the reaction mixture.

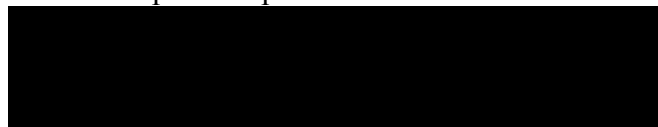
### **2.4 How does it work?**

To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on. The cycle of denaturing and synthesizing new DNA is repeated as many as 30 or 40 times, leading to more than one billion exact copies of the original DNA segment. The entire cycling process of PCR is automated and can be completed in just a few hours. It is directed by a machine called a Thermocycler, which is programmed to alter the temperature of the reaction every few minutes to allow DNA denaturing and synthesis.

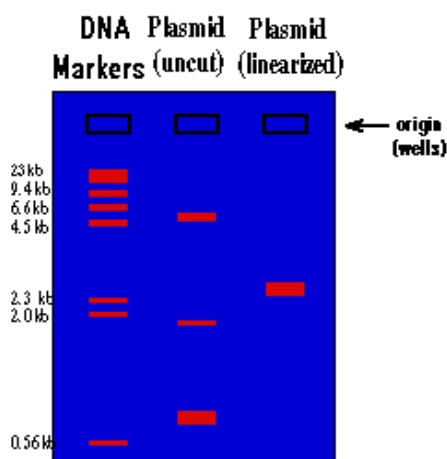
## **3 Electrophoresis**

Agarose gel electrophoresis is an easy way to separate DNA fragments by their sizes and visualize them. It is a common diagnostic procedure used in molecular biological labs.

The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this reason, when an electrical potential is placed on the DNA it will move toward the positive pole:



The rate at which the DNA will move toward the positive pole is slowed by making the DNA move through an agarose gel. This is a buffer solution (which maintains the proper pH and salt concentration) with 0.75% to 2.0% agarose added. The agarose forms a porous lattice in the buffer solution and the DNA must slip through the holes in the lattice in order to move toward the positive pole. This slows the molecule down. Larger molecules will be slowed down more than smaller molecules, since the smaller molecules can fit through the holes easier. As a result, a mixture of large and small fragments of DNA that has been run through an agarose gel will be separated by size. This is a graphic representation of an agarose gel made by "running" DNA molecular weight markers, an isolated plasmid, and the same plasmid after linearization with a restriction enzyme:



These gels are visualized on a U.V. trans-illuminator by staining the DNA with a fluorescent dye (Ethidium bromide). The DNA molecular weight marker is a set of DNA fragments of known molecular sizes that are used as a standard to determine the sizes of your unknown fragments.

If you click on the figure you will see a short movie that simulates the movement of the DNA bands through the gel. When looking at the video, note that bands of a low molecular weight move very quickly through the gel while high molecular weight bands move very slowly.

### 3.1 Interpretation

Much information can be derived from this gel. As you read the text below, refer back to figure 2.

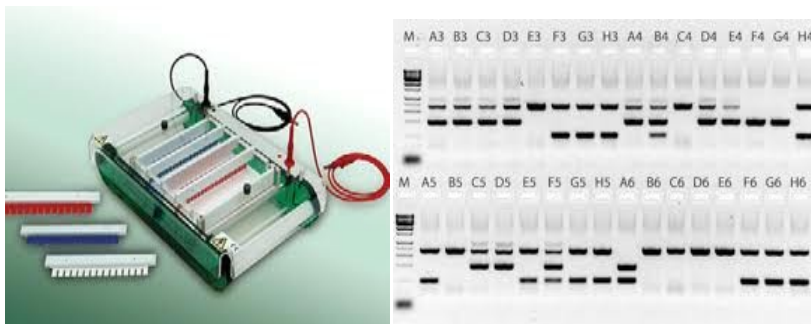
1.) By looking at the migration of the DNA molecular weight standards, you can tell that the migration of DNA through an agarose gel is not linear with respect to size. If you graphed the distance travelled vs. the molecular weight of the fragment, you would see that there is a logarithmic relationship (i.e. small fragments travel much faster than large fragments).

2.) You can see that there is a big difference between the way a plasmid as isolated from the alkaline lysis preparation will run vs. this same plasmid after it is cut with a restriction enzyme and linearized. This is because the plasmid will be found in many different supercoiled forms in the bacteria. When you isolate plasmid from a bacterial culture, you isolate all the different supercoiled forms of the plasmid, and each will migrate differently on the gel, giving you three major bands and many minor bands. When this mixture of supercoiled plasmids is cut with a restriction enzyme, the different forms linearize and unwind. As a result they all become identical and run at the same rate, and you see only one band on the gel.

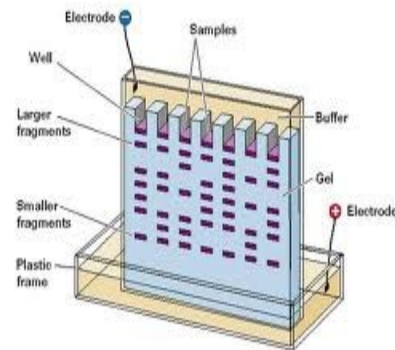
3.) The molecular size of an unknown piece of DNA can be estimated by comparison of the distance that it travels with that of the molecular weight standards. This is only true for linear DNA. None of the supercoiled forms will migrate at a rate relative to linear DNA, which means that you can't use the DNA

markers to estimate the molecular weight of a circular DNA molecule. To estimate the molecular weight of a plasmid, you must first linearize it. By looking at the gel above, the molecular size of the plasmid can be estimated at approximately 3.0 kilobases (kb). A more accurate estimate can be found by graphing the molecular weight of the standards (in base pairs) vs. the distance travelled on semi-log paper and using this graph to determine the molecular weight of the unknown. You will do this at the end of this experiment. Molecular size is the most important information derived from the agarose gel and the usual reason for running a gel.

### *Horizontal electrophoresis*



### *Vertical electrophoresis*



## **4 Restriction Fragment Length Polymorphism (RFLP)**

This is a technique in which organisms may be differentiated by analysis of patterns derived from the cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another.

This technique can be used:

- To show the genetic relationship between individuals
- To determine the relationship among species
- To analyse population polymorphisms
- To rearrange DNA molecules
- To prepare molecular probes
- To create mutants
- To analyse modification status of the DNA

### **4.1 Method PCR-RFLP**

Usually, DNA from an individual specimen is first extracted and purified. Purified DNA may be amplified by PCR. The DNA is then cut into restriction fragments by endonucleases, which only cut where there are specific DNA sequences recognized by the enzymes. The restriction fragments are then separated according to length by agarose gel electrophoresis.

### **4.2 Restriction endonucleases**



These are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular used. Enzyme recognition sites are usually 4 base pairs in length. If molecules differ in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. Restriction enzymes are isolated from a wide variety of bacterial genera and are thought to be part of the cell's defence against invading bacterial viruses. These enzymes are named by using the first letter of the genus, the first letter of the species, and the order of discovery.

### **4.3 Sites of cleavage**

Rather than cutting DNA indiscriminately, a restriction enzyme cuts only double helical segments that contain particular nucleotide sequence, and it makes its incision only within that sequence known as a recognition sequence, always in the same way.

Some enzymes make strand incisions immediately opposite one another, producing "blunt end" DNA fragments. Most enzymes make slightly staggered incisions, resulting in "sticky ends", out of which one strand protrudes.

## **5 Denaturing Gradient Gel Electrophoresis (DGGE)**

### **5.1 Introduction**

Denaturing gradient gel electrophoresis (DGGE) is a commonly used technique in molecular biology and has been a staple of environmental microbiology for characterization of population structure and dynamics. The method is a powerful one, and can rapidly provide a tangible characterization of community diversity and composition, and shifts in population can be readily be demonstrated. DGGE analyses are also used in the medical field for detection of mutations, including single nucleotide polymorphisms (SNPs). These advantages are coupled by a number of limitations and these limitations should be well understood before employing the technique. There is often a steep learning curve.

While there are a number of trials and tribulations related to the actual operation of the DGGE analysis, it is important to remember that many of the difficulties with DGGE belong to the stages prior to the DGGE. Since DGGE analyses require a significant amount of DNA for detection, a polymerase chain reaction (PCR) must be performed prior to analysis. Thus, all the troublesome features of sampling, DNA (or RNA) extraction, reverse transcription (if employing RNA extraction), PCR primer design, PCR conditions, and PCR cleanup bear some thought when troubleshooting DGGE problems. Every stage of molecular analysis can impact (often negatively) each stage downstream. However, these are considerations that are endemic to molecular biology, and indeed to experimental science as a whole.

### **5.2 Background information**

DGGE is a molecular fingerprinting method that is employed for the separation of double-stranded DNA fragments (PCR-generated DNA products) that are identical in length, but differ in sequence. The PCR environment DNA can generate templates of differing DNA sequence that represent many dominant microbial organisms. However, since PCR products from a given reaction are of familiar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results ion differential denaturation characteristics of DNA. The technique exploits (among other factors) the difference in the stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds)

### **5.3 Principles**

DDGE is based on a principle that increasing denaturants will melt double stranded DNA domains. When the melting temperature ( $T_m$ ) of the lowest domain is reached, the DNA will partially melt, creating branched molecules and reducing its mobility in a polyacrylamide gel. The denaturing environment is created by uniform run temperature between 50<sup>0</sup> C and 65<sup>0</sup> C and a linear denaturant gradient formed with urea (ranging from 0-7M) and formamide.

Complete strand separation is prevented by presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of a sequence of 40 GC.

During DGGE, PCR products of different sequence encounter increasingly higher concentrations of chemical denaturants as they migrate through a polyacrylamide gel. Upon reaching the threshold denaturant concentration, the weaker melting domains of the double-stranded DNA fragments migrate better in acrylamide gel, while denatured DNA molecules become efficiently larger and slow down or stop in gel. In this manner, DNA fragments of differing sequence can be separated in acrylamide gel.

Differing sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands. Each band theoretically representing a different bacterial population present in the community. Once generated, fingerprints can be uploaded into databases in which fingerprint similarity can be assessed to determine microbial structural differences between environments or among treatments. Furthermore, with the breadth of PCR primers available, DDGE can also be used to investigate broad phylogenies or specific target organisms such as soil bacteria and fungi, or pathogens.

### **Advantages of DGGE**

- a) High detection rate and sensitivity.
- b) DGGE gives you an overview of the diversity.
- c) DGGE gives you the opportunity to get some more sequence information by excision of bands. Not much, but often enough to give you a rough classification.
- d) With DGGE multiple samples can be tested simultaneously. Therefore, as you will have realized, DGGE is nice for time series study in one ecosystem.
- e) The methodology is simple and non- radioactive detection method used.

### **Disadvantages of DGGE**

- a) Preliminary experiments are essential when setting up DGGE from scratch
- b) Purchase of DGGE equipment may be required.
- c) Primers are more expensive because of 40 bases of GC clamp. Additional primers may be required for sequencing.
- d) Analysis of PCR fragments over 400bp is less successful.
- e) Genes which are exceptionally GC rich are not easily analysed by DGGE.
- f) Method involves the use of formamide

## **6 Selective growth media**

Much of the study of microorganisms depends on its ability to grow in the laboratory, and this is possible only if suitable culture media are available for the growth of microorganism. A culture medium is defined

as a solid or liquid preparation used for the growth, transport, and storage of microorganisms. The effective culture medium must contain all the nutrients required for the growth of the microorganism.

Specialized media are widely employed for the isolation and identification of microorganisms, testing the antibiotic sensitivities, analysis of water and food, industrial microbiology, and other activities. Although all microorganisms need sources of energy, nitrogen, carbon, phosphorus, sulfur, and various minerals, the exact composition of a satisfactory medium will rely on the species one is trying to identify and cultivate because nutritional requirements vary so greatly among the microorganisms.

Knowledge of microorganism's normal habitat is often useful in selecting a suitable culture medium because its nutrient requirements reflect its natural surroundings. A medium is used to select and growing specific microorganisms or to help identifying a particular species. In these cases, the function of the medium also depends on its composition. In addition to nutrients necessary for the growth of all bacteria, special-purpose media contain one or more chemical compounds that are essential for their functional specificity.

These include: **Selective, Differential and Enriched Media.**

### **6.1 Selective media**

Selective media allows the growth of certain type of organisms, while inhibiting the growth of other organisms. This selectivity is achieved in several ways. For example, organisms that have the ability to utilize a given sugar are screened easily by making that particular sugar the only carbon source in the medium for the growth of the microorganism. Like-wise, the selective inhibition of some types of microorganisms can be studied by adding certain dyes, antibiotics, salts or specific inhibitors that will affect the metabolism or enzymatic systems of the organisms. For example, media containing potassium tellurite, sodium azide or thallium acetate at different concentrations of 0.1 - 0.5 g/l will inhibit the growth of all gram-negative bacteria. Media supplemented with the antibiotic penicillin concentration 5-50 units/ml or crystal violet 2 mg/l inhibits the growth of gram-positive bacteria. Tellurite agar is used to select for gram-positive organisms, and nutrient agar supplemented with the antibiotic penicillin can be used to select for the growth of Gram negative organisms.

### **6.2 Differential media**

Differential media are widely used for differentiating closely related organisms or groups of organisms. Because of the presence of certain dyes or chemicals in the media, the organisms will produce certain characteristic changes or growth patterns that are used for identification or differentiation of microorganism, e.g., Mac Conkey (MCK) agar, Eosin Methylene Blue (EMB) agar

### **6.3 Enriched media**

Enriched media are media that have been supplemented with highly nutritious materials such as blood, serum or yeast extract for the purpose of cultivating fastidious organisms., e.g., Blood agar, Chocolate agar. Some of the special-purpose media are as follows:

#### **6.3.1. Mannitol Salt Agar (MSA)**

Mannitol salt agar is both a selective and differential media used for the isolation of pathogenic *Staphylococci* from mixed cultures.

On MSA, only pathogenic *Staphylococcus aureus* produces small colonies surrounded by yellow zones. The reason for this color change is that *S. aureus* have the ability to ferment the mannitol, producing an acid, which, in turn, changes the indicator color from red to yellow. The growth of other types of bacteria is usually inhibited.

This growth differentiates *S. aureus* from *S. epidermidis*, which forms colonies with red zones or both zones.

### 6.3.2. MacConkey's Agar (MAC)

MacConkey's Agar is both a selective and differential media; it is selective for gram-negative bacteria and can differentiate those bacteria that have the ability to ferment lactose.

By utilizing the available lactose in the medium, Lac<sup>+</sup> (Lactose positive) bacteria such as *Escherichia coli*, *Enterobacter* and *Klebsiella* will produce acid in the medium, which lowers the pH of the agar below 6.8 and results in the appearance of red or pink colonies. The bile salts in the medium precipitate in the immediate neighborhood of the colony, causing the medium surrounding the colony to become hazy appearance. Non-lactose fermenting bacteria such as, *Proteus species*, *Salmonella*, *Pseudomonas aeruginosa* and *Shigella* cannot utilize lactose in the medium, and will use peptone instead. This results in the formation of ammonia, which raises the pH of the agar, and leads to the formation of white or colorless colonies in the plate. But, in some cases, they can also look golden to brown with dark centers. They are usually circular colonies and arranged randomly.

### 6.3.3. Eosin Methylene Blue (EMB) Agar (Levine)

Eosin methylene blue agar (EMB) is both a selective and differential medium used for the detection and isolation of Gram-negative intestinal pathogens.

Acid production from lactose fermentation causes precipitation of the dyes on the surface of the colony resulting in different colors.

- ✓ Large amounts of acid → green metallic sheen
- ✓ Small amounts of acid → pink
- ✓ No fermentation → colorless

*Enterobacter aerogenes* produces large colonies which are pink-to-buff around dark centers. *Escherichia coli* produce small, dark colonies with a green metallic sheen. *Pseudomonas*, *Proteus*, *Salmonella* and *Shigella sp* produces colorless colonies because it does not ferment lactose.

### 6.3.4. Phenyl ethyl Alcohol Agar

Phenyl ethyl Alcohol (PEA) Agar with or without 5% sheep blood is a selective medium for the isolation of gram-positive organisms, particularly gram-positive cocci, from specimens of mixed gram-positive and gram-negative flora.

### 6.3.5. Hektoen Enteric (HE) Agar

Hektoen Enteric (HE) Agar is a moderately selective medium used in qualitative procedures for the isolation and cultivation of gram-negative enteric microorganisms, especially *Shigella* and *Salmonella* from a variety of clinical and nonclinical specimens.

### 6.3.6. Blood Agar

Blood agar is both differential and enriched medium. The blood that is incorporated into this medium is an enrichment ingredient for the cultivation of fastidious organisms such as the *Streptococcus* species.

A number of streptococcal species produce substances that destroy red blood cells; that is, they cause lysis of the red cell wall with subsequent release of hemoglobin. Such substances are referred to as hemolysins. The activity of streptococcal hemolysins also known as streptolysins can be readily observed when the organisms are growing on a blood agar plate.

Different *streptococci* produce different effects on the red blood cells in blood agar. Those that produce incomplete hemolysis and only partial destruction of the cells around colonies are called alpha-hemolytic *Streptococci*. Characteristically, this type of hemolysis is seen as a distinct greening of the agar in the hemolytic zone, and thus this group of *streptococci* has also been referred to as the viridans group.

Species whose hemolysins cause complete destruction of red cells in the agar zones surrounding their colonies are said to be beta-hemolytic. When growing on blood agar, beta-hemolytic streptococci are small opaque or semi translucent colonies surrounded by clear zones in a red opaque medium. Two types of beta lysins are produced: Streptolysin O and Streptolysin S. Streptolysin O, an antigenic, oxygen-labile enzyme, and streptolysin S, a nonantigenic, oxygen-stable lysin. The hemolytic reaction is enhanced when blood agar plates are streaked and simultaneously stabbed to show subsurface hemolysis by Streptolysin O in an environment with reduced oxygen tension. Some strains of *Staphylococci*, *Escherichia coli*, and other bacteria also may show beta-hemolysis.

Some species of *Streptococci* do not produce hemolysins. Therefore, when their colonies grow on blood agar, no change is seen in the red blood cells around them. These species are referred to as nonhemolytic or gamma hemolytic streptococci.

On blood agar, *S. aureus* usually displays a light to golden yellow pigment, whereas *S. epidermidis* has a white pigment and *S. saprophyticus* either a bright yellow or white pigment. However, pigmentation is not always a reliable characteristic. On blood agar, *S. aureus* is usually, but not always, beta-hemolytic; *S. epidermidis* and *S. saprophyticus* are almost always nonhemolytic.

### 6.3.7 Chocolate Agar

Fastidious organisms such as *Haemophilus* and *Neisseria* require specially enriched culture media and microaerophilic incubation conditions. “Chocolate” agar is commonly used for primary isolation of *Haemophilus* from clinical specimens. This medium contains hemoglobin derived from bovine red blood cells as well as other enrichment growth factors. Chocolate agar may be made selective for *Haemophilus* species by the addition of bacitracin.

**Table:** A summary of growth media types

Media	Purpose
<b>Complex</b>	Grow most heterotrophic organisms
<b>Defined</b>	Grow specific heterotrophs and are often mandatory for chemoautotrophs, photoautotrophs and for microbiological assays
<b>Selective</b>	Suppress unwanted microbes, or encourage desired microbes
<b>Differential</b>	Distinguish colonies of specific microbes from others
<b>Enrichment</b>	Similar to selective media but designed to increase the numbers of desired microorganisms to a detectable level without stimulating the rest of the bacterial population
<b>Reducing</b>	Growth of obligate anaerobes

## 7 Mycorrhiza

### 7.1 What are arbuscular Mycorrhizal fungi?

Latin Myco=fungus; rhiza=root

The term “Mycorrhiza” was coined by A. B. Frank, a researcher in Germany, more than 100 years ago. It means “fungus-root,” and stands for the mutualistic association existing between a group of soil fungi and

higher plants. Mycorrhiza fungi (AMF) are important biotrophic organisms, which live in symbiosis with approximately 80% of land plants, forming a Mycorrhiza (i.e., a root colonized by a symbiotic fungus). These associations should not be confused with rhizobial associations, which are symbiotic associations with bacteria that result in nitrogen-fixing nodules.

Two major groups of fungi form Mycorrhizal associations: ectomycorrhizal fungi and endomycorrhizal fungi. Ectomycorrhizae associate mainly with temperate-zone trees such as pine, poplar, and willow and some non-nodulating tropical legumes in Cealapinaceae such as *Afzelia*, *Brachystegia*, *Julbernardia*, *Paramacrolobium* amongst others, *Uapaca* and *Dipterocarpaceae*. These fungi form distinct sheaths around their host's root surfaces and are easily distinguished from endomycorrhiza. Mycorrhizal associations are symbiotic associations that benefit both plant and fungal partners. While the plants supplies photosynthates such as sugar to the fungi, the fungi are multi-purpose, accomplishing many essential functions for their plant partner, the most important being their role in the absorption of nutrients from the soil. For most land plants, Mycorrhiza are the main means of obtaining nutrients from the soil and we can consider the Mycorrhizal condition as the norm more than an exception for the plant. Most ectomycorrhizae can be grown in pure culture.

## 7.2 Ectomycorrhizae (ECM)

Unlike AM fungi, many ECM fungi show a high level of host specificity and associate with dominant tree species in forest ecosystems, playing a key role in the natural regeneration of these trees. ECM fungi are responsible for many of the fruit bodies found under trees in the forests. ECM roots normally occur near the soil surface in the litter layers and are easily seen with the naked eye as the root is distinctly swollen and the fungus surrounding the tree root is often brightly colored. The distinctive features of a typical ectomycorrhiza are the fungal mantle that surrounds the host root, and the labyrinthine fungal hyphae that penetrate between the epidermal or cortical cells to form a network known as the Hartig net. From the mantle surface, hyphae or bundles of interwoven hyphae (rhizomorphs) radiate out into the surrounding soil. In tropical forests, rhizomorph development can be prolific, sometimes extending for several meters from the tree root.

One of the main benefits of ECM fungi to the host tree is their ability to break down litter and access inorganic and organic sources of nitrogen and phosphorus. These rhizomorphs are therefore able to efficiently explore large volumes of soil, which is of paramount importance in nutrient deficient soils. Besides being important in nutrient uptake and water absorption, ECM fungi can also increase the tolerance of trees to attack by root pathogens, to soil toxins, to drought and to extremes of soil temperature and pH.

Over 5,000 species of fungi have been recorded as forming ectomycorrhizae. Most ECM fungi are Basidiomycetes or Ascomycetes, typically producing large, fleshy fruit bodies such as mushrooms, toadstools, puffballs, truffles, coral fungi, cup fungi and resupinate fungi. Unlike AM taxonomy, which depends on the characterization of individual soil-borne spores, the taxonomy of these complex, organized fruit bodies is well established and can provides firm corroboration of the identity of ECM species below ground.

Endomycorrhizae are distinguished to arbuscular Mycorrhizal fungi (AMF) formerly known as the vesicular arbuscular Mycorrhizal (VAM) fungi, which form associations with most plants (approximately 80 per cent of all plant species) amongst them commercially important crops. These fungi cannot be grown in pure culture but must be grown in association with plant roots. They all form branched structures called arbuscules within the host's root cells, and thus they are known as arbuscular Mycorrhizal fungi, while others form vesicles. The arbuscules are sites of nutrient exchange between the

fungus and the host and the vesicles are storage sites from starch. Other types of Mycorrhiza are the ericoid mycorrhiza, which is specific to the Ericaceae family and the orchid Mycorrhiza specific to Orchidaceae family.

### 7.3 Arbuscular Mycorrhiza (AM)

AM are by far the most widespread Mycorrhizal type and tend to dominate many plants growing in dry tropical regions. The main benefit to the plant of AM association is improved growth through enhanced phosphorus uptake. Phosphorus, water and other nutrients are translocated to the plant roots via an extensive hyphal network formed by AM fungi which extends from the plant roots into the soil. This means that large volumes of soil can be explored for nutrients at a very low C cost to the plant. Low phosphorus availability is typical of most tropical soils and many plants are entirely dependent on their AM for survival and sustained growth. The low mobility of phosphorus causes faster depletion at the root zone and the roots cannot access phosphorus beyond the depletion zone. The Fungal hyphae extend beyond this depletion zone and into crevices where roots cannot penetrate to capture more phosphorus. All nutrients of low mobility such as Zinc and ammonium are largely enhanced by Mycorrhiza.

AM fungi are not host specific but have preferences and form Mycorrhizal associations with a wide range of plant species. However, they may differ in effectiveness. Most tree legumes also form root nodules with nitrogen-fixing bacteria (Rhizobia) and effective nodulation and Mycorrhizal association enables them to sustain growth in both phosphorus and nitrogen deficient soils.

Arbuscular Mycorrhizal exist outside the root as spores and mycelia and inside the root (vegetative form) as **coiled hyphae, arbuscules** and **vesicles**. The vegetative structures are mostly found inside the root cortical cells, and are formed after the AM fungal hyphae have penetrated the root epidermal cells by forming structures called appressoria. Once inside the root, the fungal hyphae spread by passing from cell to cell or by growing through intercellular spaces. The coiled hyphae, arbuscules and vesicles formed inside the cells are separated from the cytoplasm by a plasma membrane across which nutrients and photosynthates are transferred. These Mycorrhizal structures do not alter the overall appearance of the roots and, unlike the ectomycorrhizal type of association, AM roots cannot be distinguished from non-mycorrhizal roots with the naked eye. Because of this, AM roots need to be processed and stained before AM structures can be observed and the level of Mycorrhizal infection in the roots can be determined under the microscope.

The arbuscules are sites of nutrient exchange between the fungus and the host. The associations that arbuscular Mycorrhizal fungi form with plants are called symbiotic associations because they are usually beneficial to both organisms. In exchange for carbohydrates produced by the host through photosynthesis, the fungi help the plant take up water and immobile soil nutrients such as phosphorus (P), copper, and zinc. The fungus extends from the plant root and expands the volume of soil that the root system can explore by itself. To have beneficial associations between the fungus and plant roots, a low but sufficient level of P in the soil or rooting medium is needed. If the soil P level is extremely low, the fungus can be parasitic (harmful to the plant) rather than beneficial, because it will compete with the plant for available P. When soil P is high (above “sufficient”), the plant can obtain enough P without the fungus, and the association will not be formed.

### 7.4 Why plants should be inoculated with arbuscular Mycorrhizal fungus?

In many tropical soils, P availability is limited due to P fixation. Plants inoculated with arbuscular Mycorrhizal fungi in the nursery are better able to obtain P when they are later planted into low-P soils. In addition, the association helps the plant obtain water, which is critical to plant survival and growth under

dry conditions. The amount of P fertilizer that needs to be applied to a plant or crop is reduced when effective arbuscular Mycorrhizal associations have been formed. In addition to the benefit of lowering fertilizer costs, reducing P applications can help maintain environmental water quality. Erosion of soil from fields with high P levels

## 7.5 AM fungi identification

AM fungi can be identified by examining the large, asexual spores which they produce in the soil. Features such as spore wall layers, hyphal attachments and germination structures are used to characterize the different species. AM fungi were formerly classified as lower fungi (Zygomycota) because of their asexual mode of reproduction and the predominance of aseptate hyphae. However, studies of spore development and recent advances in molecular characterization now place AM fungi in their own separate phylum (Glomeromycota) and have also meant that current generic and species delineations are in a state of flux. Nevertheless, extraction and identification of AM spores from the soil still appears to be the most reliable method of comparing AM populations in different soils.

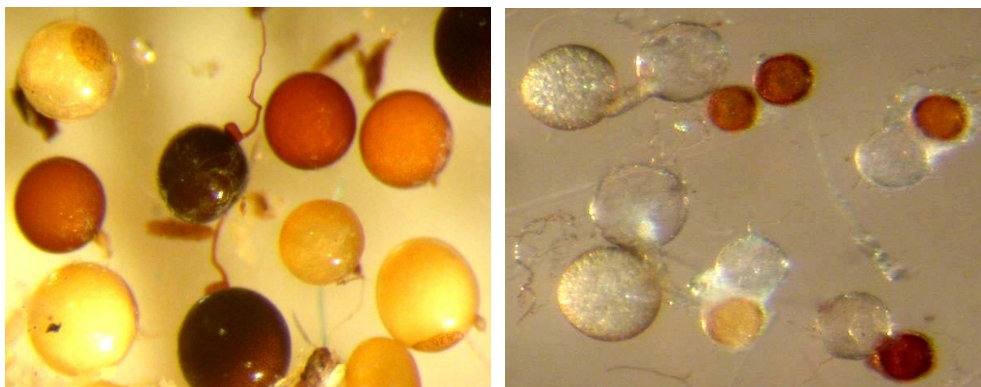
AM fungi are obligate symbionts and can only be propagated by growing them on the living roots of a host plant. For establishment of known cultures of AM fungi, it is necessary to obtain clean, viable spores of the target species in order to initiate colonisation of non-mycorrhizal seedlings. Production of AM inoculum therefore requires that a fast growing host plant (usually a crop species) is grown in a soil medium where only the inoculant fungus is present. As a consequence, the soil medium used for the production of AM inoculum has to be sterilised first to remove all other contaminant fungi.

### 7.5.1 Preparation of diagnostic slides of AM spores

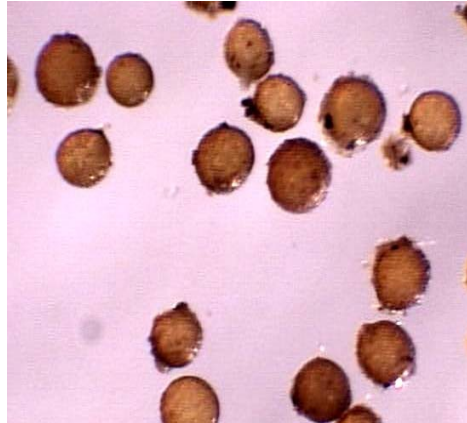
Permanent slides of AM spores should be prepared. Spores are squashed on microscope slides to reveal details of spore wall layers and hyphal attachments. All preparatory stages are carried out under a dissecting microscope. Clean and separated similar spores mounted using needle (minimum 10 large spores: minimum 30 small spores). Spores are examined under a compound microscope for wall layers, hyphal feature and staining reaction in Melzer's.

### 7.5.2 Characterization and identification of AM spores

General features such as colour, size, spore development, spore aggregation and hyphal attachments can be observed under the dissecting microscope.





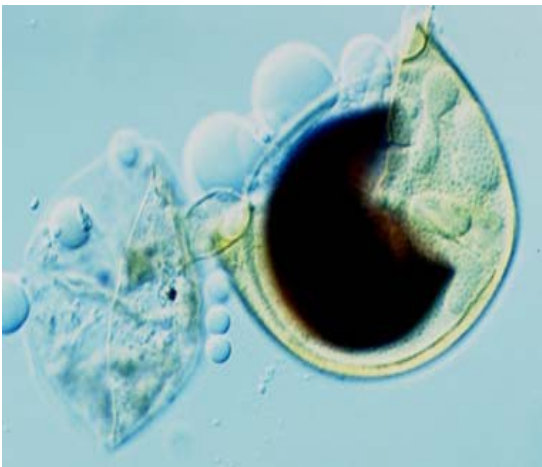


1. **Parasitized and dead spores** need to be recognised so they can be disregarded. Look for shrunken/collapsed walls, lack of cytoplasm and evidence of parasitism such as pinholes in walls or darkened contents.
2. **Detailed descriptions** of spore features are obtained by examining diagnostic slides under the compound microscope.
3. Features of **wall layers** such as ornamentation, thickness, texture and reaction to Melzer's reagent can be examined.
4. Other wall structures associated with **spore germination** can be observed such as germination shields and tubes.
5. Features of the **subtending hyphae and saccules** can be examined such as suspensor cells, hyphal shape and septation, pore closure, saccule attachments/scars, auxiliary cells.

NB/

Before identification is possible, some species may need to be **isolated and cultured** so that spore development can be examined.

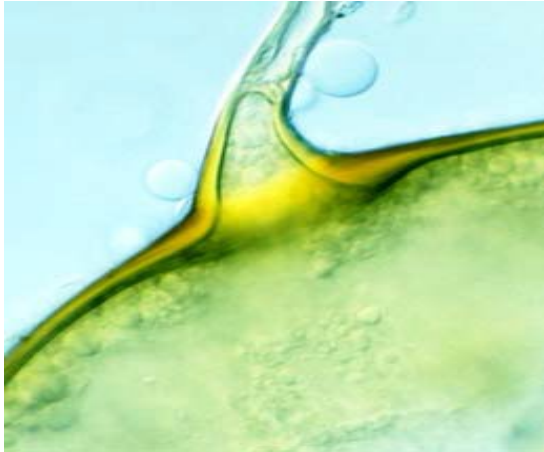
Examples of spores



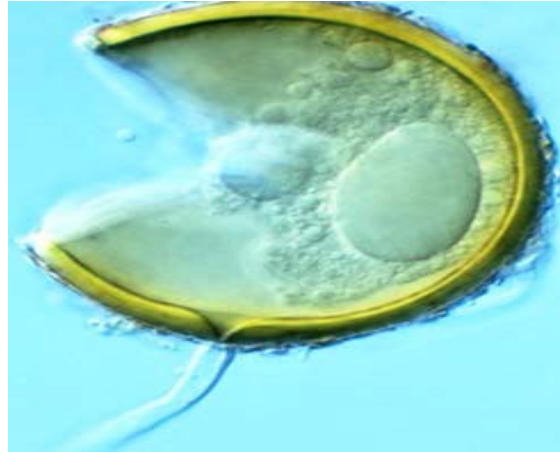
*Acaulospora* sp.



*G. clarum*



*G. mosseae*



*G. etunicatum*

## 7.6 AMF root infection

Before AMF colonization can be observed, root samples need to be taken through a series of stages during which roots are cleared in potassium hydroxide, bleached in alkaline hydrogen peroxide, acidified in hydrochloric acid and stained in trypan blue. This procedure describes different procedures for visualizing the colonization of roots by the arbuscular Mycorrhizal fungi (AMF). Dead and active AMF structures cannot be differentiated but the method provides contrastive staining allowing easy quantification and the frequency of the extent of colonization (percentage of root length and percentage of roots colonized by the AMF).

### 7.6.1 Features of AM root infection

Under the dissecting microscope, intracellular structures such as arbuscules and coiled hyphae and hyphae are difficult to differentiate; only intraradical spores and vesicles are easily identified. AM fungal hyphae in the soil penetrate the root by forming appressoria on the epidermal cells or enter via the root hairs. Auxilliary cells formed by *Gigaspora* spp. (spiny) or *Scutellospora* spp. (nodulose) often remain attached to the root by hyphae after staining. Arbuscules are the sites of nutrient and sugar exchange between plant and fungus, and arbuscular development is often most intense in the innermost cortical cells, nearest the endodermis. Coiled hyphal structures develop throughout the cortical cells, and can also occur in the epidermal cells. Coiled hyphae often develop smaller side branches and may have a similar function to the arbuscules. Vesicle development often occurs later in the development of AM infection. Vesicles are filled with lipids and may have a function in storage, survival and reinfection of new roots. Vesicles are not formed by *Gigaspora* spp. or *Scutellospora* spp. Those formed by *Acaulospora* spp. are typically thin-walled and irregularly shaped.

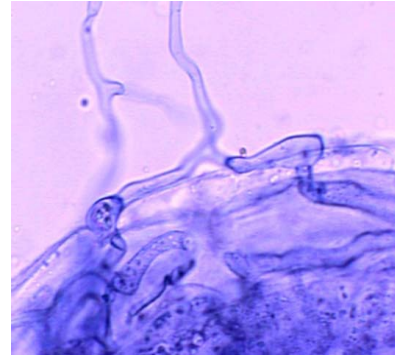
Root infection by other non-mycorrhizal endophytes can be mistaken for AM infection. Some examples of infection by commonly occurring dark, septate hyphae, intracellular zoospores and microsclerotia.



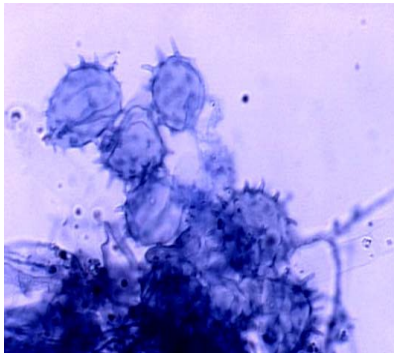
Vesicles, intraradical spores



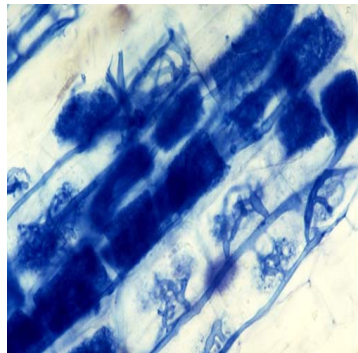
Hyphae, coiled hyphae, arbuscules



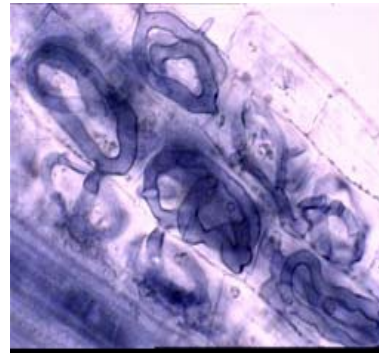
Appressoria, hyphal entry into root cells



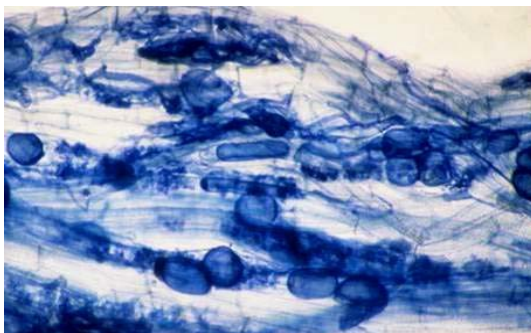
Auxilliary cells



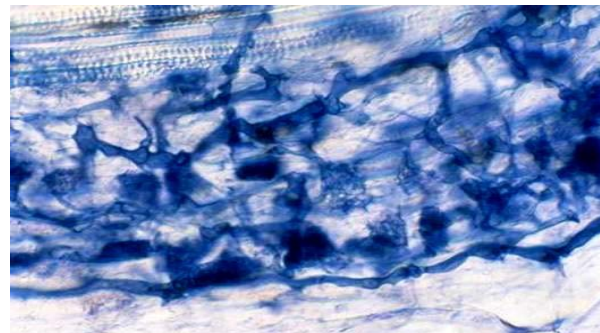
Arbuscules



Coiled hyphae



Intense hyphal, vesicle development



Intense hyphal, arbuscular development

## 7.7 Trap Cultures and Inoculation strategies for AM fungi

Identify sites such as natural forest or a site where the target plant species is well established and growing well. Collect soil and roots from under the tree species. 'Trap cultures' are used to increase the viability and activity of AM propagules such as spores, infected root fragments and hyphae present in the soil. Trap culturing is also used to eliminate pathogens and pests present in the field soil. The resulting cultures can be used as inoculum containing a mixture of species or can be used to produce single species cultures. Isolation of single species cultures is difficult and requires large numbers of fresh, viable spores. Trap culture inoculum contains an unknown mixture of AM fungi, and therefore growth benefits cannot be assigned to a particular AM fungal species. However, inoculated plants infected with a mixture of AM fungi may be able to adapt better to a range of different planting sites. Single species cultures enable the testing and selection of the best AM fungi for use as inoculants.

### 7.7.1 Determining the abundance of infective propagules in crude inoculum and in soil

Determining the number of infective propagules in soil and crude inoculum can be complex for various reasons. First, fungal structures such as spores, vesicles, arbuscules, mycelium, and even colonized roots act as infective propagules. Secondly, AMF cannot be cultured under in vitro conditions apart from their host plants. Although spores can be isolated and counted, not all of them are ready to germinate, and hence spore numbers are often not strongly correlated with AMF infectivity. The most reliable method of assessing the number of infective AMF propagules contained in a crude inoculum, soil, or sheared Mycorrhizal roots is the most-probable-number (MPN) technique, which permits a statistical estimation of microbial population density without a direct count of single cells or colonies. The MPN technique is the most precise method to estimate Mycorrhizal propagule numbers because it considers the infectivity of viable spores, mycelial fragments, and fragments of colonized roots.

### **7.7.2 Procedures**

The technique is based on determining the presence or absence of microorganisms in several individual aliquots of each of several consecutive dilutions of a sample of soil or other materials containing microbial propagules. A serial dilution, usually 10-fold, of a soil or crude inoculum sample is prepared using sterile sand, soil, or sand-soil mixture as the diluent. From each dilution, a predetermined amount of material is used to inoculate each of cups containing sterile soil or sand-soil mixture optimized for Mycorrhizal activity with a soil-solution P concentration of 0.02 mg/L. Germinated seeds or seedlings of a suitable Mycorrhizal plant (onion, clover, leucaena, etc.) are sown in these cups, which are placed in a reservoir containing water or P-free nutrient solution. Indicator plant of choice for MPN determination is *Leucaena leucocephala*, and it is grown on a 1:1 mansand:soil mixture. The P concentration of the medium is 0.02 mg/L and its pH is 6.2. The plants are then allowed to grow in the greenhouse or growth chamber for four weeks. At the end of the growth period, the roots are excised, washed, cleared, and stained. The stained roots are spread in a Petri dish and scored for the presence or absence of AMF colonization. Do not count detached hyphae or germinated spores. To calculate the most probable number of infective propagules in a sample, the statistical table is essential (Habte and Osorio, 2001).

## **8. Characterization of isolated microorganism**

### **8.1 Gram Staining**

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decoloring process.

#### **8.1.1 Principle**

The cationic dye crystal violet is used to stain the nucleic acids of the micro-organisms and background tissues. The crystal violet staining is then laked with iodine, forming a black complex. Certain micro-organisms resist differentiation due to the impermeability of their cell walls. However, using a suitable differentiator, (eg alcohol, aniline, or acetone), the tissue background and certain species of micro-organisms lose their staining, but take up a cationic dye of contrasting colour (usually red) subsequently applied.

### 8.1.2 How Does Gram Staining Work?

Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolorization, and counterstaining, usually with safranin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria, Gram positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolorization process, while Gram negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process. The process involves three steps:

- Cells are stained with crystal violet dye. Next, a Gram's iodine solution (iodine and potassium iodide) is added to form a complex between the crystal violet and iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water.
- A decolorizer such as ethyl alcohol or acetone is added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. The large crystal violet-iodine complex is not able to penetrate this tightened peptidoglycan layer, and is thus trapped in the cell in Gram positive bacteria. Conversely, the outer membrane of Gram negative bacteria is degraded and the thinner peptidoglycan layer of Gram negative cells is unable to retain the crystal violet-iodine complex and the color is lost.
- A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram positive cells. However, the decolorized Gram negative cells are stained red.

### 8.2 Oxidase Test

The oxidase test determines whether a microbe can oxidize certain aromatic amines, for example, *p*-aminodimethylaniline, to form colored end products. This oxidation correlates with the cytochrome oxidase activity of some bacteria, including the genera *Pseudomonas* and *Neisseria*. The oxidase test identifies organisms that produce the enzyme cytochrome oxidase.

The oxidase reagent contains a chromogenic reducing agent, which is a compound that changes color when it becomes oxidized. If the test organism produces **cytochrome oxidase**, the oxidase reagent will turn blue or purple within 15 seconds. Cytochrome oxidase participates in the electron transport chain by transferring electrons from a donor molecule to oxygen.

While a positive oxidase test is important in the identification of these genera, the test is also useful in characterizing the enteric bacteria (*Enterobacteriaceae*), which are oxidase-negative. Avoid using cultures which are too young or too old because they may give inaccurate results (refer to TSBF SOP-MI12/V01).

**NOTE: This test is for Gram-negative organisms.**

### 8.3 Catalase Test

The catalase test facilitates the detection of the enzyme catalase in bacteria. It is essential for differentiating catalase-positive *Micrococcaceae* from catalase-negative *Streptococcaceae*. While it is primarily useful in differentiating between genera, it is also valuable in speciation of certain gram positives such as *Aerococcus urinae* (positive) from *Aerococcus viridians* (negative) and gram-negative

organisms such as *Enterobacteriaceae*, *Campylobacter fetus*, *Campylobacter jejuni*, and *Campylobacter coli* (all positive) from other *Campylobacter* species. The catalase test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the enzyme. In this context, the catalase test is valuable in differentiating aerotolerant strains of *Clostridium*, which are catalase negative, from *Bacillus*, which are catalase positive (Mahon, Lehman and Manuselis, 2011).

The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide (Wheelis, 2008). Catalase expedites the breakdown of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) into water and oxygen ( $2\text{H}_2\text{O}_2 + \text{Catalase} \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ). This reaction is evident by the rapid formation of bubbles

#### **8.4 Culturing on media**

The acclaimed Rhizobia in the inoculants are cultured on YEMA (Yeast Extract Mannitol Agar) while the other non-rhizobial inoculants were cultured on NA (Nutrient Agar). NA is a non selective media and allows the growth of most common microorganisms. The NA cultures are placed in an incubator at 37°C since this temperature is suitable for the growth of the main expected strains (*Bacillus*, *Paenibacillus*, *Azospirillum*, *Pseudomonas*...). YEMA is a slightly selective mannitol based medium that favors the growth of rhizobia. A few microorganisms (e.g. Rhizobia) are able to utilize mannitol as their first source of carbohydrates. Most of the other microorganisms growth are inhibited or slowed down and the growth of rhizobia is promoted. YEMA cultures are placed in an incubator at 28°C which is the optimized temperature for rhizobial growth (Atieno, 2012).

The inoculants are serial diluted in sterile physiological water (9 g/l NaCl) up to  $10^{-8}$  and 0.1 ml of each dilution poured and evenly spread on a plate containing media. The plates are incubated at the optimized temperature and observed daily to record the growth and the macroscopic aspect of every colony. This included the shape, outline, texture, size, colour, opacity... Different bacterial colonies that grow on the same media are separated and re-cultured by streaking on the same media and incubated at the optimal temperature. The isolated colonies are then purified three times on the same media. Pure bacterial cultures are finally cultivated in liquid media and stored in 20% glycerol at -80°C for subsequent analyses (TSBF SOP-MI05/V01; SOP-MI11/V01).

### **9 Quantification of cells contained in the commercial products**

Quantification can be done by various techniques such plate counting and Malassez cell techniques, which majored on previously. Plate counting has been described as the best available method for determining viable number of cells that are capable of growth on a given medium under the set of conditions used for incubation (Claus, 1989). With this method, one is able to quantify separately the different types of bacteria contained in an inoculant (Reasoner and Geldreich, 1985) but some of the bacteria might be lost if they are not viable or cannot grow in these particular conditions (Atieno, 2012). On the other hand, Malassez cell counts both viable or live and dead cells without introducing a bias to the bacteria culture (Atieno, 2012). It however does not discriminate among the different types of bacteria present in a suspension (Sakr *et al.*, 2009). These two techniques can be used together in order to balance the pros and cons associated with both methods.

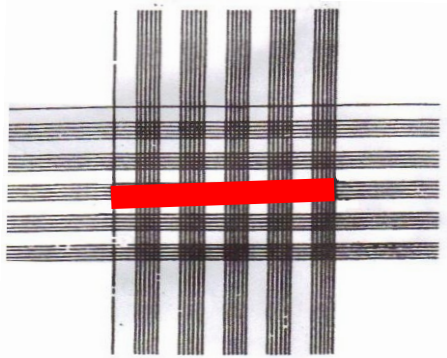
#### **9.1 Plate counting**

Plate counting method involves preparation of a dilution series from a broth culture or carrier, spreading or dropping an aliquot of the appropriate dilution to the surface of a nutrient medium, then counting the

resulting colonies after a period of incubation in optimal conditions for the targeted strains (Claus, 1989). With the number of colonies, the spread volume and the dilution, it is possible to estimate the bacteria concentration of the initial suspension. For protocol on plate counting, refer to TSBF SOP-MI10/V01.

## 9.2 Malassez cell technique

The Malassez cell is a slide with an engraved lines network. The network is composed of 100 rectangles into 10 lines. Each rectangle is composed of 20 squares and the total volume of the network is 1 mm<sup>3</sup> (Sakr *et al.*, 2009). Counting is done on one line, as seen on the scheme, in triplicates using a counter.



**Fig.** Lines network on a Malassez slide

To be sure not to count the same cells twice when they are on the edge engraved marks, the cells are counted on the marks at the top and on the right side of the square or line only. For example, if the counting is done on the middle line, the green cells were counted, and the red ones were not. For more details on Malassez slide counting, refer to TSBF SOP-MI10/V01.

The lines represent a 0.1 mm<sup>3</sup> volume each so the formula used in the calculation is (Pettipher *et al.*, 1980):

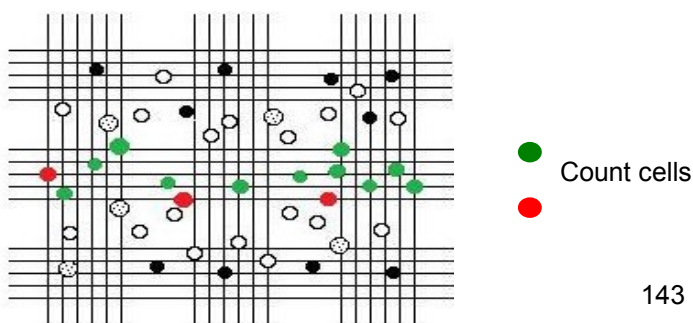
$$N = \frac{n \times 10^3}{C \times v}$$

N: cellular concentration (number of cells per ml)

n: total number of cells counted

v: studied volume in mm<sup>3</sup> (number of lines where the cells are counted x 0.1)

C: dilution used



**Fig.** Cells as seen on a Malassez slide

## 10 Phylogenetic analysis

Bacterial identifications are now usually done using their 16S rRNA gene sequences. The reasons for using the 16S rRNA gene are twofold:

- First, the 16S rRNA gene is sufficiently distinct from its eukaryotic, archaeal, mitochondrial and chloroplastic homologs, so that each can be amplified separately. It is however enough conserved so that almost universal bacterial amplification can be performed using specific primers.
- Second, the 16S rRNA gene sequence is generally species specific: the microbiologist or clinical researcher cannot only determine the diversity within a bacterial community after alignments and classification (or phylogeny), but the sequence itself allows generally for species determination (with a few counter examples).

There are two usual applications:

- One wants to study the microbial community within a given environmental or clinical sample. The vast majority of environmental bacteria cannot be cultured in the laboratory while but their DNA can be easily extracted. From this DNA, identification using the 16S rRNA gene sequences is usually performed, after cloning the PCR products and random sequencing of tens to thousands of clones.
- A strain has been isolated, and one wishes to know if it is a well-known species or a new one.

In both cases, the common approach is to blast the new sequence(s), align it to the most similar sequences found, and do a classification or a phylogenetic analysis in order to decide about identification. Even when doing biodiversity analyses, it is generally best not only to find out which are the most similar sequences present in the public databases, but also which well-known cultured species is closest (in order to have an idea of which kind of biochemical process they can contribute to, for example).

### 10.1 Some basics Terminology

**Alignment:** Arranging of sequences of DNA/RNA/Protein to identify regions of similarity that MAY be related to function or evolution of those sequences

**Clustering:** Assignment of cases to groups according to a specific procedure. Uses a calculated coefficient and linkage algorithm



A phylogenetic tree is composed of branches (edges) and nodes. Branches connect nodes; a node is the point at which two (or more) branches diverge. Branches and nodes can be internal or external (terminal). An internal node corresponds to the hypothetical last common ancestor (LCA) of everything arising from it. Terminal nodes correspond to the sequences from which the tree was derived (also referred to as operational taxonomic units or 'OTUs'). Trees can be made up of multigene families (gene trees) or a single gene from many taxa (species trees, at least theoretically) or a combination of the two. In the first case, the internal nodes correspond to gene duplication events, in the second to speciation events.

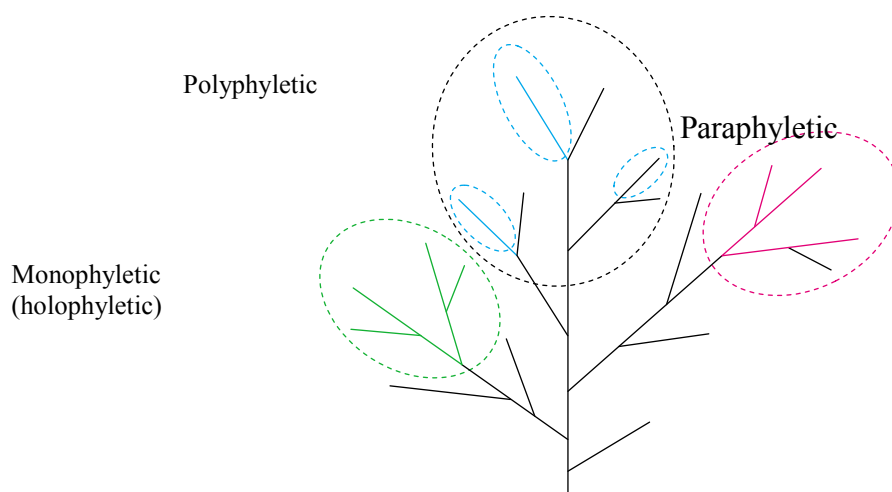
## 10.2 Groups

Trees are about groupings. A node and everything arising from it is a 'clade' or a 'monophyletic group'. A monophyletic group is a natural group; all members are derived from a unique common ancestor (with respect to the rest of the tree) and have inherited a set of unique common traits (characters) from it. A group excluding some of its descendants is a paraphyletic group (e.g. animals excluding humans). A hodge-podge of distantly related OTUs, perhaps superficially resembling one another or retaining similar primitive characteristics, is polyphyletic; that is, not a group at all.

## 10.3 Trees

Intuitively we draw trees from the ground up like real trees. However, as these trees get larger and more complex, they can become cluttered and difficult to read. As an alternative we can expand the nodes and turn the tree on its side. Now the tree grows left to right, and all the labels are horizontal. This makes the tree easier to read and to annotate. Thus, the widths of the nodes have no meaning; they are simply adjusted to give even spacing to the branches. To make things slightly more complicated, all branches can rotate freely about the plane of their nodes, so all trees in Fig. 2 are identical (except that tree F is 'unrooted', see below).

Molecular phylogenetic trees are usually drawn with proportional branch lengths; that is, the lengths of the branches correspond to the amount of evolution (roughly, per cent sequence difference) between the two nodes they connect. Thus, the longer the branches the more relatively divergent (highly evolved) are the sequences attached to them. Alternatively, trees can be drawn to display branching patterns only ('cladograms'), in which case the lengths of the branches have no meaning, but this is rare done with molecular sequence trees.



**Fig.** Trees are about groups: monophyletic (holophyletic), paraphyletic and 'polyphyletic'.

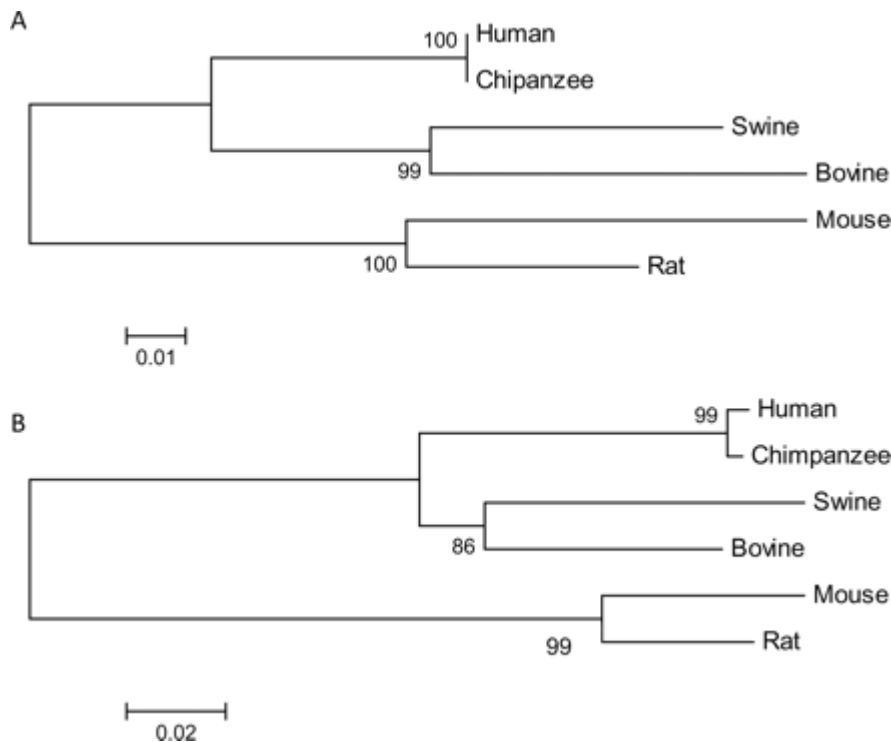
### 10.4 Bootstrapping

So how good was that tree? The simplest test of phylogenetic accuracy is the bootstrap; it is rare now to see a tree without it. Bootstrapping essentially tests whether your whole dataset is supporting your tree, or if the tree is just a marginal winner among many nearly equal alternatives. This is done by taking random subsamples of the dataset, building trees from each of these and calculating the frequency with which the various parts of your tree are reproduced in each of these random subsamples. If group X is found in every subsample tree, then its bootstrap support is 100%, if it is found in only two-thirds of the subsample trees, its bootstrap support is 67%. Each of the subsamples is the same size as the original, which is accomplished by allowing repeat sampling of sites; that is, random sampling with replacement. It is a simple test, but bootstrap analyses of known phylogenies (viral populations evolved in the laboratory) show that it is a generally dependable measure of phylogenetic accuracy, and that values of 70% or higher are likely to indicate reliable groupings.

### 10.5 Data presentation

Finally, there are few set rules on how to present phylogenetic trees, but there are some widely accepted conventions. In molecular phylogenetic trees, branch lengths are almost always drawn to scale; that is, proportional to the amount of evolution estimated to have occurred along them. Although the relationship between branch lengths and real time is far

from straightforward and probably unreliable for any single gene, lengths still give a good general impression of relative rates of change across a tree. Bootstrap values should be displayed as percentages, not raw values. This makes the tree easier to read and to compare with other trees. By convention, only bootstrap values of 50% or higher are reported; lower values mean that the node in question was found in less than half of the bootstrap replicates. Example



**Fig:** Phylogenetic tree representing the evolutionary relationship of six mammalian *IL23A* (A) and *IL12B* (B) mRNA sequences. Sequences were aligned using ClustalW and a neighbour-joining phylogenetic tree was generated using the maximum composite likelihood method and MEGA4. The tree is drawn to scale and the scale bar represents the same units as the evolutionary distance used to infer the phylogenetic tree; base substitutions per site. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. (A) The human *IL23A* transcript (*H. sapien*; NM\_016584) is genetically identical to the predicted transcript of the chimpanzee (*P. troglodytes*; XM\_522436). The next closest evolutionary related species is the swine (*S. scrofa*; NM\_001130236), followed by the bovine (*B. taurus*; XM\_588269), rat (*R. norvegicus*; NM\_130410) and finally the mouse (*M. musculus*; NM\_031252). The scale bar represents a genetic distance calculated by the number of base substitutions per site of 0.01. (B) The human *IL12B* transcript (NM\_002187) is highly similar to the predicted transcript of the chimpanzee (XM\_527101). The next closest evolutionary-related species is the swine (NM\_214013), followed by the bovine (NM\_174356), rat (NM\_022611) and finally the mouse (NM\_008352). The scale bar represents a genetic distance calculated by the number of base substitutions per site of 0.02.

## 11 Rhizobia

**Rhizobia** are soil bacteria that fix nitrogen (diazotrophs) after becoming established inside root nodules of legumes (Fabaceae). Rhizobia require a plant host; they cannot independently fix nitrogen. In general, they are Gram-negative, motile, non-sporulating rods. The first species of rhizobia, *Rhizobium leguminosarum*, was identified in 1889, and all further species were initially placed in the *Rhizobium* genus. However, more advanced methods of analysis have revised this classification, and now there are many in other genera. Most research has been done on crop and forage legumes such as clover, alfalfa, beans, and soy; recently, more work is occurring on North American legumes.

The word *rhizobia* comes from the Ancient Greek, *rhíza*, meaning "root" and, *bios*, meaning "life". The word *rhizobium* is still sometimes used as the singular form of *rhizobia*

### 11.1 Infection and signal exchange

The symbiotic relationship implies a signal exchange between both partners that leads to mutual recognition and development of symbiotic structures. Rhizobia live in the soil where they are able to sense flavonoids secreted by the roots of their host legume plant. Flavonoids trigger the secretion of nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses, such as ion fluxes. The best-known infection mechanism is called intracellular infection, in this case the rhizobia enter through a deformed root hair in a similar way to endocytosis, forming an intracellular tube called the infection thread. A second mechanism is called "crack entry"; in this case, no root hair deformation is observed and the bacteria penetrate between cells, through cracks produced by lateral root emergence. Later on, the bacteria become intracellular and an infection thread is formed like in intracellular infections.

The infection triggers cell division in the cortex of the root where a new organ, the nodule, appears as a result of successive processes.

### 11.2 Nodule formation and functioning

Infection threads grow to the nodule, infect its central tissue and release the rhizobia in these cells, where they differentiate morphologically into **bacteroids** and fix nitrogen from the atmosphere into a plant-usable form, ammonium ( $\text{NH}_4^+$ ), using the enzyme nitrogenase. In return, the plant supplies the bacteria with carbohydrates, proteins, and sufficient oxygen so as not to interfere with the fixation process. Leghaemoglobins, plant proteins similar to human hemoglobins, help to provide oxygen for respiration while keeping the free oxygen concentration low enough not to inhibit nitrogenase activity. Recently, a *Bradyrhizobium* strain was discovered to form nodules in *Aeschynomene* without producing nod factors, suggesting the existence of alternative communication signals other than nod factors.

The legume–rhizobium symbiosis is a classic example of mutualism—rhizobia supply ammonia or amino acids to the plant and in return receive organic acids (principally as the dicarboxylic acids malate and succinate) as a carbon and energy source, but its evolutionary persistence is actually somewhat surprising. Because several unrelated strains infect each individual plant, any one strain could redirect resources from nitrogen fixation to its own reproduction without killing the host plant upon which they all depend. But this form of cheating should be equally tempting for all strains, a classic tragedy of the commons. There are two competing hypotheses for the mechanism that maintains legume-rhizobium symbiosis (though both may occur in nature). The **sanctions hypothesis** suggests the plants police cheating rhizobia. Sanctions could take the form of reduced nodule growth, early nodule death, decreased carbon supply to nodules, or reduced oxygen supply to nodules that fix less nitrogen.

The **partner choice hypothesis** proposes that the plant uses prenodulation signals from the rhizobia to decide whether to allow nodulation, and chooses only noncheating rhizobia. There is evidence for sanctions in soybean plants, which reduce rhizobium reproduction (perhaps by limiting oxygen supply) in nodules that fix less nitrogen. Likewise, wild lupine plants allocate fewer resources to nodules containing less-beneficial rhizobia, limiting rhizobial reproduction inside. This is consistent with the definition of sanctions just given, although called "partner choice" by the authors. However, other studies have found no evidence of plant sanctions, and instead support the partner choice hypothesis.

Many other species of bacteria are able to fix nitrogen (diazotrophs), but few are able to associate intimately with plants and colonize specific structures like Legume nodules. Bacteria that do associate with plants include the actinobacteria *Frankia*, which form symbiotic root nodules in actinorhizal plants, and several cyanobacteria (*Nostoc*) associated with aquatic ferns, *Cycas* and *Gunneras*. Free-living diazotrophs are often found in the rhizosphere and in the intercellular spaces of several plants including rice and sugarcane, but in this case the lack of a specialized structure results in poor nutrient transfer efficiency compared to legume or actinorhizal nodules.

## 12 Fusarium

*Fusarium* is a large genus of filamentous fungi widely distributed in soil and in association with plants. Most species are harmless saprobies, and are relatively abundant members of the soil microbial community. Some species produce mycotoxins in cereal crops that can affect human and animal health if

they enter the food chain. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes.

### 12.1 Introduction to the genus

The genus has over 50 species including many plant pathogens and toxin producers. The basic concept to *Fusarium* species concepts: Morphological species, Biological species and phylogenetic species. out of 101 most economically important plants, 81 have at least one plant associated with *Fusarium* disease, along with the fact that each *Fusarium* species keeps its own toxicological profile, it is a challenge to ascertain the taxonomic status of *Fusarium* species on their phenotypical characteristics (including pathogenicity and toxigenicity) alone.

The main approach for the *Fusarium* classification is still morphology, and the primary trait for species to be placed in *Fusarium* genus is the occurrence of the asexual spores, the distinctive banana-shaped macroconidia, firstly diagnosed by Link. *Fusarium* species produce three types of spores: macroconidia, microconidia and chlamydospores. Septated macroconidia can be produced on monophialides and polyphialides in the aerial mycelium, but also on short monophialides in specialized structures called sporodochia. A mono- phialide is a conidiation cell with a unique pore from which the endoconidia are released; a polyphialide can possess several such openings. Microconidia can vary in shape and size, and are produced in the aerial mycelium in clumps or chains, both on monophialides and polyphialides. Finally, hlamydospores

are resistance structures with thickened walls and high lipid content; in the case of their presence, they can form in the middle of the hyphae or at their termini. The different shape of acroconidia remains the most important feature for distinguishing the species. Moreover, other traits, such as the presence/absence of microconidia and their shape, the presence/absence of chlamydospores, and the characteristics of the micro- and macro-conidiogenous cells, contribute to distinguishing species in *Fusarium*. In order to identify the species, all taxonomists suggest the use of strain cultures derived from single-spore isolation, and growing the strains on special media under standard incubation conditions.

## 13 Identification of *Trichoderma* spp.

### 13.1 Introduction

*Trichoderma* is a soil fungi which reproduces asexually which is frequently isolated and most prevalently culturable. It is widely distributed in plant material, decaying vegetation, wood and other diverse habitats (Ranasingh, 2006). *Trichoderma* spp. are facultative anaerobes, grow saprophytically or as parasites on other *Trichoderma* and are able to grow in soils having a pH range of 2.5 - 9.5, although most of them prefer moderately acidic environment (Grondona, 1997). Most of the *Trichoderma* strains produce only asexual spores. However, a few strains have sexual stage which is not considered for biocontrol purposes. In recent past, morphological features were considered to determine different taxonomical aspects with the help of the asexual sporulation apparatus, but molecular approaches are now being used.

The strains show a high level of genetic diversity which can be used to produce various products of commercial and ecological interests. They are prolific producers of extra-cellular proteins, and also known to produce enzymes that degrade cellulose and chitin. Many other different strains produce more than 100 different metabolites having antibiotic activities. Since the introduction of biocontrol, *Trichoderma* species have been recognized as agents for the control of plant diseases and for their roles in increasing plant growth and development. The most useful strains show a property that is known as 'rhizosphere competence' that is, the ability to colonize and grow in association with plant roots. Much of the known biology and many of the uses of these fungi have been documented recently (Harman,

2000). To isolate *Trichoderma* spp. different media are used. Some selective media are more efficient than others. Depending on the species, *Trichoderma* can show no growth to broadly spreading growth on Potato Dextrose Agar media and Czapek's agar.

### 13.2 Identifying Characteristics

- Facultative anaerobes
- Grows saprophytically or as a parasite on other fungi
- Among most common saprophytic fungi
- As parasites, they grow toward hyphae of other fungi, coil around them, and attach to host mycelium

### 13.3 Preservation and Distribution of Fungal Cultures

Maintaining and preserving fungal cultures are essential elements of systematics and biodiversity studies. Because fungi are such a diverse group, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological, and genetic integrity of the cultures over time. The cost and convenience of each method, however, also must be considered.

The primary methods of culture preservation are continuous growth, drying, and freezing. Continuous growth methods, in which cultures are grown on agar, typically are used for short-term storage. Such cultures are stored at temperatures of from 5°-20°C, or they may be frozen to increase the interval between subcultures. The methods are simple and inexpensive because specialized equipment is not required. Drying is the most useful method of preservation for cultures that produce spores or other resting structures. Silica gel, glass beads, and soil are substrata commonly used in drying. Fungi have been stored successfully on silica gel for up to 11 years (Smith and Onions 1983). Drying methods are technically simple and also do not require expensive equipment. Freezing methods, including cryopreservation, are versatile and widely applicable. Most fungi can be preserved, with or without cryoprotectants, in liquid nitrogen or in standard home freezers. With freeze drying, or lyophilization, the fungal cultures are frozen and subsequently dried under vacuum. The method is highly successful with cultures that produce mitospores. Freeze-drying and freezing below -1 35°C are excellent methods for permanent preservation, and we highly recommend them. However, both methods require specialized and expensive equipment, as described in the next section (see "Liquid Nitrogen" and "Lyophilization" under "Long-term Preservation," later in this chapter).

The choice of preservation method depends on the species of concern, the resources available, and the goal of the project. Some low- cost methods of preservation, such as storage in distilled water and the silica gel method, are good, but none is considered permanent. The maximum duration of storage varies with each method and with the species being preserved, but it generally is 10 years or less. Whenever possible, fungal strains should be preserved with one of the permanent methods (lyophilization, cryopreservation) described later in this chapter see "Long-term Preservation"). Permanent preservation is essential for strains with critically important characteristics and for type specimens. Cultures that are permanently preserved in metabolically inactive states now can serve as type specimens, according to Article 8.4 of the International Code of Botanical Nomenclature (Greuter et al. 2000).

#### 13.3.1 Maintenance and preservation of cultures

## Short-term preservation

Short-term preservation involves maintenance of cultures for up to 1 year. Most fungal cultures can be maintained for that period by serial transfer. The method is simple, inexpensive, and widely used, although time consuming and labor intensive, periodic transfer is a good option for small collections with cultures in constant use for short periods (less than 1 year). The method also has several disadvantages, however. Cultures must be checked frequently for contamination by mites or other microorganisms and for drying. In addition, the morphology and physiology of a cultured fungus may change over time. In particular, the ability to sporulate or to infect a host may be lost after repeated transfers. Because of those disadvantages, the technique is generally inappropriate for long-term (more than 1 year) preservation of cultures. Inoculum is transferred from an actively growing fungus culture to test tubes (screw cap or plugged with cotton or foam) or Petri dishes (wrapped with Parafilm to reduce drying) containing an agar medium of choice; Alternating nutrient-rich with nutrient-poor media at each transfer helps to maintain healthy cultures.

Some fungi, such as endophytic and entomopathogenic species, have specific media requirements (Bacon 1990; Singleton et al. 1992; Humber 1994). After a culture is established, it is kept at room temperature or at 4°C. Cultures must be checked periodically for contamination and desiccation. Fungi such as oomycetes and some basidiomycetes (e.g., *Boletus*, *Coprinus*, *Cortinarius*, and *Mycena*) should be transferred monthly if kept at 16°C (von Arx and Schipper 1978). Most filamentous fungi can survive at least 1-2 years at 4°C. Vigorous, sporulating cultures also can be sealed tightly and stored in a freezer at -20°C (Carmichael 1956, 1962) or stored at -70°C (Pasarell and McGinnis 1992) to enhance survival and increase the interval between required transfers (see

## Long-term preservation

### (i) Sclerotization

Some fungi develop sclerotia or other long-term survival propagules in culture as well as in nature; preserving such structures, usually at 3°-5°C, is a good way to preserve fungal strains. Sclerotia and spherules of various myxomycetes have been germinated successfully after 1-3 years of storage. Many soil fungi, such as *Magnaporthe*, *Phymatotrichum*, and *Cylindrocladium* species, produce sclerotia or microsclerotia that remain viable for 2-5 years (Singleton et al. 1992). Instructions for inducing formation of spherules, sclerotia, and microsclerotia are available in Daniel and Baldwin (1964) and Singleton and colleagues (1992). Sometimes rice straw or toothpicks are used as substratum to promote sclerotia production in culture. Jump (1954) described a simple method for inducing sclerotium formation in *Physarum* species. A piece of sterile cellophane cut to the dimensions of a Petri dish is placed over a dish containing 1% water agar. An actively growing plasmodium is then transferred to the cellophane and allowed to grow overnight. The cellophane is removed from the agar; placed in a sterile, dry Petri dish; covered; and allowed to dry for 24 hours. The Petri dish lid is then removed to allow the sclerotia to air-dry until brittle. The cellophane is cut into small pieces, each of which is stored in its own screw-cap vial. Alternatively, the sclerotia are removed from the cellophane and stored in a vial.

### (ii) Oil Overlay

A low-cost and low-maintenance method for preserving cultures growing on agar slants is oil overlay. Cultures can be kept for several years or, in exceptional cases, up to 32 years at room temperature or 15°-20°C. This method is appropriate for mycelial or nonsporulating cultures that are not amenable to freezing or freeze-drying. As an added benefit, oil also reduces mite infestations. Although many basidiomycetes can be maintained this way, the growth rates of the cultures slow as storage times increase (Johnson and Martin 1992; Burdsall and Dorworth 1994). The major disadvantage of the oil overlay

technique is that the fungi continue to grow, and thus, selection for mutants that can grow under adverse conditions may occur.

High-quality mineral oil or liquid paraffin is sterilized by autoclaving at 15-lb (6.8-kg) pressure for 2 hours. Entrapped moisture is removed by heating the liquid in a drying oven at 170°C for 1-2 hours (optional). Fungal cultures grown on agar slants are covered with about 10 mm of oil or paraffin. The entire agar surface and fungal culture should be submerged completely in the oil. The tubes are kept in an upright position at room temperature (15°-20°C; 12°C for *Pythium* species and *Phytophthora* species; G. Adams, personal communication). The oil level in the tubes or vials must be checked periodically, and more oil should be added, if necessary.

To retrieve a culture from mineral oil, a small amount of the fungal colony is removed and placed on appropriate media after as much oil as possible has been drained. Lifting the Petri dish on one side to form a slight angle often helps the oil drain. It may be necessary to subculture the colony several times to get a vigorous oil-free culture.

### **(iii) Immersion in Distilled Water**

Another inexpensive and low-maintenance method for storing fungal cultures is to immerse them in distilled water. Apparently, the water suppresses morphological changes in most fungi. The method has been used successfully to preserve oomycetes. The procedures used for covering cultures on agar slants with oil also can be used when covering them with sterile distilled water. Alternatively, sterilized straws or Pasteur pipettes (large-diameter end) are used to cut disks from the growing colony edge. The disks are transferred to sterile cotton-plugged or screw-cap test tubes filled with several millilitres of water. To save space, small (1.8 ml), sterile, screw-cap cryovials are filled with several discs and topped with sterile distilled water. Test tubes (loosely capped and wrapped with Parafilm) are stored at room temperature; tightly capped tubes and vials are stored at 4°C. Disks are removed aseptically and transferred to fresh agar medium to retrieve cultures. An alternative method for sporulating fungi (McGinnis et al. 1974) involves inoculating agar slants of preferred media with fungal cultures and then incubating them at 25°C for several weeks to induce sporulation. Sterile distilled water (6-7 ml) is added aseptically to the culture, and the surface of the culture is scraped gently with a pipette to produce a spore and mycelial slurry. This slurry is removed with the same pipette and placed in a sterile, 2-dram glass vial (or cryovial). The cap is tightened, and the vials are stored at 25°C. To retrieve a culture, 200-300 µl of the suspension is removed from the vial and placed on fresh medium.

### **(iv) Organic Substrata**

Over the years, researchers have developed practical, effective, and ingenious methods of preserving fungi on various organic substrata such as wood chips, cereal grains, and straw, filter paper, and insect and plant tissues. Many of the techniques were developed for pathogenic or other specific fungi and have not been rigorously tested with a range of fungi.

**Wood-** Wood-inhabiting fungi can be successfully stored on wood chips or toothpicks as long as the colony is growing vigorously (Nelson and Fay 1985; Delatour 1991; Singleton et al. 1992). Some wood-inhabiting basidiomycetes and ascomycetes can be stored on wood chips for up to 10 years. If the fungi do not vigorously colonize the wood chips, however, the method fails. Small pieces of untreated beech wood (12-mm diameter × 6-mm thick) are added to 2% malt-extract broth (about 60 pieces of wood per 100 ml broth; and sterilized for 20 minutes at 121°C (Delatour 1991). The mixture is sterilized again 24 hours later. About 15 wood chips are drained and placed on a colony of the fungus that is growing on malt extract agar in Petri dishes. The Petri dishes are sealed with Parafilm, and the fungus is allowed to



colonize the wood chips. After 10-15 days, the inoculated wood chips are transferred to sterile test tubes (18 × 180 mm) containing 6-7 ml of 2%-malt agar. The tubes are plugged with cotton and incubated for about 1 week, after which time the cotton is replaced with sterile Parafilm and aluminum foil. The tubes are stored at 4°C. To retrieve a culture, a piece of wood chip is removed and placed on fresh agar medium. The tube is resealed and returned to the refrigerator.

**Cereal Grains-** Fungi such as *Sclerotinia*, *Magnaporthe*, *Leptosphaeria*, and *Rhizoctonia* species have been stored for up to 10 years on seeds of oats, barley, wheat, rye, millet, and sorghum (Singleton et al. 1992). To preserve isolates of *Rhizoctonia* species, barley, oat, or wheat grains (Sneh et al. 1991) are soaked overnight in water containing chloramphenicol (250 g/ml). The water is removed, and the grain is autoclaved for 1 hour at 12°. Cover 2 consecutive days. Screw-cap vials are filled with the grain and autoclaved. The vials are inoculated with transfers from the margins of actively growing cultures and incubated at 23°-27°C for 7-10 days. The cultures then are dried thoroughly in a desiccation chamber. The caps are tightened and wrapped with Parafilm, and the vials are stored at 25°C.

**Agar Strips-** Nuzum (1989) described a method of vacuum-drying fungal cultures on agar strips. *Pythium*, *Rhizoctonia*, and some basidiomycete species survived 18 months with this method, whereas ascomycetes and their mitosporic forms survived from 3-5 years. Fungal cultures are grown on appropriate media in Petri dishes. Strips 1-cm long are cut from the growing edge of the colony and placed in sterile Petri dishes. After 1 week at room temperature, the pieces of dried agar are transferred to sterile ampoules, vacuum-dried, and sealed. To revive cultures, agar strips are placed on fresh medium of choice.

**Insect or Plant Tissue-** The host tissue can be used as a substrate on which to maintain and store cultures of some pathogenic fungi. For example, roots of plants infected with *Pyrenochaeta* and *Thielaviopsis* can be dried and then frozen (Singleton et al. 1992). *Neozygites fresenii* cannot be cultured in vitro, but Steinkraus et al. (1993) developed a method of preserving viable conidia on frozen, infected aphid mummies.

**Soil or Sand** -Some fungi can be preserved easily and successfully for many years in dry, sterile soil or sand. This low maintenance and cost-effective method is appropriate for fungi such as *Rhizoctonia* (Sneh et al. 1991), *Septoria* (Shearer et al. 1974), and *Pseudocercospora* (Reinecke and Fokkema 1979). Dormancy caused by dryness can take time to develop, however, and morphological changes in some fungi have been recorded. Glass bottles (60 ml) are filled to two-thirds capacity with sand or loam soil (water content 20%) and then sterilized by autoclaving for 20 minutes at 120°C. The bottles are allowed to cool and then sterilized again. Sterile, distilled water is added to a culture, and the colony surface is scraped gently to produce 5 ml of spore or mycelial suspension. One millilitre of the suspension is added to each bottle of soil or sand. After 2-14 days of growth at room temperature, the bottles are capped loosely and stored in the refrigerator at 4°C. To retrieve the fungus, a few grains of soil are sprinkled onto fresh agar medium. Test tubes or vials can be used in place of glass bottles to save space.

**Silica Gel-**The silica gel method can be used to preserve sporulating fungi if facilities for freeze-drying or for storage in liquid nitrogen are not available. It originally was developed by Perkins (1962) for *Neurospora* species. He found that sporulating fungi protected by skim milk and stored on silica gel remain viable for 4-5 years. Spores and microcysts of dictyostelids can be preserved for up to 11 years on silica gel (Raper 1984). In general, viability after storage on silica gel depends on the strain of fungus and the medium on which it was grown before storage. When cultures are stored in soil, the initial growth period before storage may permit variant vegetative strains to develop and overgrow the wild type, or saprotrophic segregants to overgrow pathogenic ones. The advantage of silica gel is that it prevents all

fungal growth and metabolism. Some researchers use glass beads instead of silica gel. Revival of cultures from silica gel is easy—a few silica gel crystals are scattered on an agar plate. The same storage container can be used for successive sampling. The Fungal Genetic Stock Center has used this technique successfully since 1962 for preserving genetic stocks of *Aspergillus nidulans* and *Neurospora crassa*. Fungi such as *Pythium* and *Phytophthora* species, however, do not survive this process.

#### (v) Freezing

Most fungal cultures frozen at  $-20^{\circ}$  to  $-80^{\circ}\text{C}$  in mechanical freezers remain viable. Freezing with liquid nitrogen is discussed in the following section. Cultures grown on agar slants in bottles or test tubes with screw caps can be placed directly in the freezer (Carmichael 1956). Overall failure rate for mitosporic ascomycetes, Zygomycetes, and yeasts after 5 years in storage at  $-20^{\circ}\text{C}$  was 5.1% (Carmichael 1962). The failure rate of medically important fungi, aerobic actinomycetes, and algae stored from 6 months to 13 years at  $-70^{\circ}\text{C}$  was 2.3% (Pasarell and McGinnis 1992). Ito (1991) and Ito and Yokoyama (1983) aseptically removed six 6-mm disks from vigorously growing cultures of nonsporulating basidiomycetes and ascomycetes and placed them in sterile cryotubes containing 10% glycerol in water. The cultures were preserved successfully for up to 5 years by mechanical freezing at  $-80^{\circ}\text{C}$ . Fungi grown on various organic substrata, such as cereal grains, agar strips, plant parts, and filter paper, and then dried can be frozen. In general, vigorously growing and sporulating cultures survive the freezing process better than less vigorous strains. We do not recommend repeated freezing and thawing, which will significantly reduce viability of the cultures.

#### (vi) Liquid Nitrogen

Storage in liquid nitrogen is an effective way to preserve many, if not most, organisms, including those that cannot be lyophilized. It costs somewhat more than lyophilization, however, because liquid nitrogen must be replenished every few days. Liquid-nitrogen storage is recommended for the preservation of dictyostelids. Ascomycetes that sporulate poorly in culture and higher basidiomycetes that generally grow only as mycelia in culture also can be stored in liquid nitrogen.

Because the rates of mutation in cultured fungi are likely to correspond to those of cell division and metabolic activity, storage methods that stop cell division completely and totally arrest metabolism, while still retaining viability, are best. Freezing at or below  $-139^{\circ}\text{C}$ , the temperature at which ice crystals do not grow and rates of other biophysical processes are too slow to affect cell survival, accomplishes this. All fungi can be cryopreserved, but this method generally is reserved for fungi that do not sporulate in culture, fungi that have large or delicate spores that will not survive freeze-drying, genetic stocks, and dangerous human pathogens. In addition, many culture collections, such as the American Type Culture Collection (ATCC), store their seed stock in liquid nitrogen so that when distribution stock is depleted, the material used for replenishment will be as genetically close to the original deposit as possible.

Because living cells can be damaged severely by freezing and thawing, chemical cryoprotectants are used in most protocols. Cryoprotectants are of two types: penetrating agents such as glycerol and dimethyl sulfoxide (DMSO), which readily pass through the cell membrane and protect intracellularly and extracellularly, and non-penetrating agents such as sucrose, lactose, glucose, mannitol, sorbitol, dextran, polyvinyl-pyrrolidone, and hydroxyethyl starch, which exert their protective effect external to the cell membrane. Glycerol and DMSO have proved to be most effective for fungi, although polyethylene glycol, another penetrating agent, can be used also (Ohmasa et al. 1992).

The major advantages of liquid nitrogen storage include prevention of increased genetic variability of distributed culture stocks; timesaving, reduced labor requirements compared to handling of living stocks;

elimination of the need for repeated pathogenicity tests, prevention of culture loss from contamination; and increased assurance of long-term availability of cultures. The major disadvantages of this technique are the relatively high cost of the apparatus and the liquid nitrogen that must be replaced every 2 days, the space requirement for refrigeration units, and the need for constant surveillance. Culture collections, such as the ATCC, usually maintain a backup off-site liquid-nitrogen storage facility. One disadvantage affecting distribution of strains preserved in liquid nitrogen is that they first must be grown on agar or in liquid medium to avoid the expense of shipping frozen materials.

Storage in the vapor phase of the liquid-nitrogen freezer is an attractive alternative to immersion in the liquid nitrogen. Tubes that are immersed must be sealed carefully to prevent entry of liquid nitrogen. A tube filled with liquid nitrogen that is quick-thawed at 37°C is likely to explode from the pressure created by the expanding nitrogen. Another concern is that cultures in leaky vials may be contaminated with bacteria or spores that may be present in the liquid-nitrogen freezer. Various alternative techniques for liquid-nitrogen storage, such as using plastic straws instead of vials or tubes, have been reported (Stalpers et al. 1987; Kirsop 1991). Other protocols such as the one used by Gulya et al. (1993) to preserve zoosporengia of downy mildew for up to 4 years in liquid nitrogen require neither cryoprotectants nor controlled freezing regimens.

Procedures used to harvest materials for preservation in liquid nitrogen differ depending on whether the fungus sporulates, has mycelia that penetrate below the surface of the agar, or grows only in liquid culture. Samples of human pathogens are scraped from the agar surface, or agar plugs are cut from the cultures. Such samples are never macerated in a mechanical blender because of the hazard of aerosol dispersion of the pathogen.

**Protocol A.** For fungal cultures that do not sporulate or that produce mycelia that grow deep into the agar, sterilized 2-ml screw-cap polypropylene vials are filled with 0.5-1.0 ml sterile 10% glycerol. Plugs 4mm in diameter are cut from vigorously growing cultures using a sterilized plastic straw. Several plugs are placed in the vial, the cap is tightened, and the tube is placed directly into the vapor phase (temperature is about -170°C) of a liquid-nitrogen tank. The accession number should be written on each cryovial with a cryoresistant-ink lab marker, printed onto paper, and then taped to the vial, or it should be printed onto a special cryoresistant adhesive label, which is readily available from biotechnology supply companies. The location of storage in the freezer must be indexed for rapid retrieval. Frozen preparations are retrieved by removing the vials from the freezer and rapidly thawing them in a 37°C water bath. The thawed agar plugs are placed on appropriate agar plates. Viability of the cultures should be checked from 2-7 days after storage.

**Protocol B.** To make suspensions of spores or mycelial fragments from cultures growing on the surface of agar slants or plates, the colony surface is flooded with 10% glycerol or 5% DMSO and gently scraped with a pipette.

**Protocol C.** The mycelium of a fungus that grows only in liquid culture must be macerated before it can be pipetted into vials. The broth culture is fragmented for a few seconds in a sterile mini-blender and mixed with equal parts of 20% glycerol or 10% DMSO to give a final concentration of 10% glycerol or 5% DMSO, respectively. The mixture is then treated as described in the following section.

**General Protocol.** The fungal mycelial and spore suspension is pipetted in aliquots of 0.5 ml into sterile 2-ml screw-cap polypropylene vials. Filled vials are placed into prelabeled cans in racks that then are put into the freezing chamber of a programmable freezer and allowed to equilibrate at 4°C for about 10 minutes. They are then cooled from 4° to 40°C at a rate of 1C° per minute and from -40° to -90°C at 10C°

per minute. After reaching  $-90^{\circ}\text{C}$ , vials are transferred immediately to liquid-nitrogen vapor at  $-150^{\circ}$  to  $-180^{\circ}\text{C}$ . Polypropylene vials are not immersed in liquid nitrogen.

Cultures are thawed rapidly by placing vials in a warm water ( $37^{\circ}$ - $55^{\circ}\text{C}$ ) bath until the last trace of ice dissipates. Cultures in glass ampoules and straws thaw in less than a minute; those in polypropylene containers take longer. Culture samples are then transferred aseptically to appropriate growth media.

### (vii) Lyophilization

Lyophilization, or freeze-drying, a low-cost form of permanent preservation, is not appropriate for all fungi. In fact, the technique is used primarily with species that form numerous, relatively small propagules. However, Croan (2000) demonstrated that mycelial isolates of basidiomycetes can be lyophilized effectively in the presence of trehalose. Lyophilized spores of dictyostelids, with associated bacteria, were maintained successfully for up to 30 years (Raper 1984). This procedure is the preservation method of choice for many spore-forming fungi that produce large numbers of spores  $10\text{-}\mu\text{m}$  or less in diameter. Larger spores tend to collapse during the lyophilization process, and the structural damage caused is not reversible by hydration. A significant number of the spores of appropriate size also are physically damaged and killed during the freezing process by the formation of ice crystals. Thus, each ampoule initially must contain many viable spores. Rapid freezing and the addition of a menstruum that dissolves ice crystals minimize growth of ice crystals. The two most common menstrua are non-fat dry milk powder (sterile 5% or 10% solution) and filter-sterilized bovine serum, although other proteinaceous materials also can be used.

**Equipment and Supplies.** Material required for lyophilization include the following: high-quality

mechanical vacuum pump (e.g., Edwards two-stage pump); vacuum gauge; vacuum manifold; cold trap; hoses to connect pump, trap, and manifold; insulated bath; support stand for manifold; oxygen-gas torch; oxygen supply; 10-cm lengths of 6-mm soft glass tubing with one end heat sealed, or lyophilization ampoules; cotton for plugging tubes; Pasteur pipettes; mechanical or electrical pipetting aid; sterile menstruum; and permanent ink suitable for writing on glass.

**General Protocol.** An agar slant with medium that supports good growth and sporulation is inoculated with the organism, which is allowed to grow until it reaches the resting phase. Lyophilized preparations from cultures much younger or older than resting phase often exhibit very low postlyophilization viability. Five or more lyophilization tubes are sterilized and labeled for immediate use. About 1.5-2.0-ml sterile menstruum is added to an agar slant; spores are suspended in the menstruum by gently scraping the agar surface with a Pasteur pipette. If sporulation has been light, the menstruum-spore mixture is transferred to a second agar slant, whose spores are added to the mixture. Approximately  $200\ \mu\text{l}$  of the spore suspension is added to each of several lyophilization tubes. Tubes are: plugged loosely with cotton, the open end of the glass is lubricated with castor oil, and the tubes are placed on the vacuum manifold. The manifold is lowered until the lyophilization tubes are immersed in a dry ice and ethylene glycol bath that is maintained between  $40^{\circ}\text{C}$  and  $-50^{\circ}\text{C}$  while the contents of each tube freezes (about 5 minutes). A vacuum is applied to the system for about 30 minutes while the bath warms to about  $0^{\circ}\text{C}$ . The manifold then is raised to remove the tubes from the solvent bath. Drying of the lyophilization preparations continues at room temperature until the pressure in the system reaches about 30 milliTorrs. Evaporative cooling keeps the samples frozen during the drying process. The tubes then are sealed under vacuum using a gas-oxygen torch. Finished lyophilization ampoules are stored in numbered plastic boxes or sealed plastic bags in a  $4^{\circ}\text{C}$  refrigerator. The purity and viability of the preparation in one lyophilization

vial should be checked 1-2 weeks after preservation. Viability can be roughly categorized as poor (10-50 colonies/ampoule), moderate (50-100 colonies/ampoule), or good (100-1000 colonies/ ampoule).

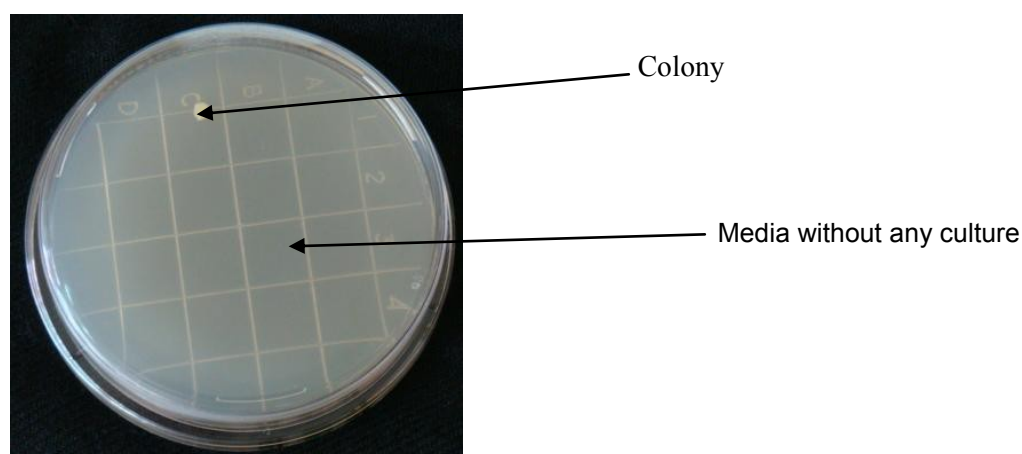
Bunse and Steigleder (1991) described an alternative method for the lyophilization of cultures grown on agar slants in glass ampoules that preserves the fungus as well as the macro morphology of the cultures. Good results were obtained with *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Mucor*, *Candida*, and *Rhodotorula* species but not with any dermatophyte species.

### 13.5 Records and record keeping

For a small research collection (i.e., fewer than 500 cultures) the least costly, yet effective, method of keeping records is through the use of index card files. Fungus collection records can be kept on three sets of 3" x 5" index cards. One set of cards is arranged alphabetically by species name; the other two sets are arranged by accession number. In this way one could readily look up species using the alphabetical file and quickly identify cultures labeled with their accession numbers using the numerical file. The cards are database kept for many years and include information on fungal strains. The third set of cards is used as an inventory of vials of lyophilized specimens and specimens stored in liquid nitrogen available for distribution. Given the common availability of personal computers and the low cost of database programs, it is now easy to enter the records for even small culture collections into electronic databases. The primary considerations when choosing a computer and a database program are universality, or inter-compatibility. Most of the common commercial (and some shareware) database programs can export data in a standard format (e.g., comma delimited fields) or a system data format. If a database can export data in one of those formats, the data can be transported to practically any database platform at will.

## 14 Surface and air control tests

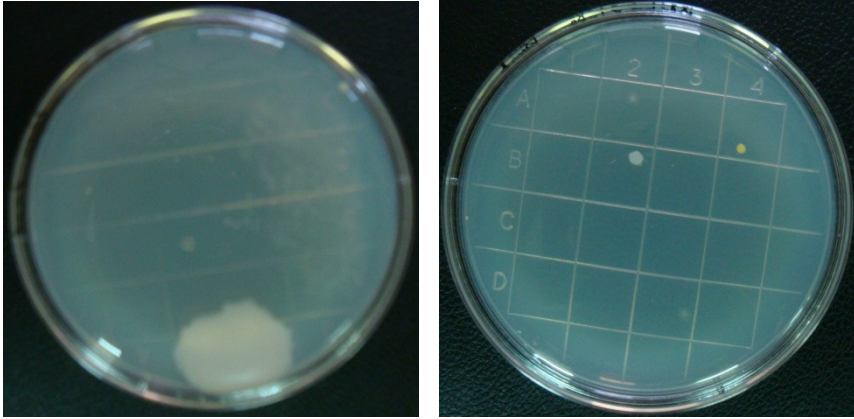
These tests are essential in ensuring the basic sterility of the lab in order to obtain valid results. These tests are carried using NA media, which allows the growth of most of the microorganisms. The cultures are incubated at 37°C for 48 hours, and then kept at room temperature for 24 more hours to allow the growth of other microorganisms, and fungus. Sampling is done using contact plates for 10 sec, under a uniform pressure.



### 14.1 Example of a report on surface and air control tests

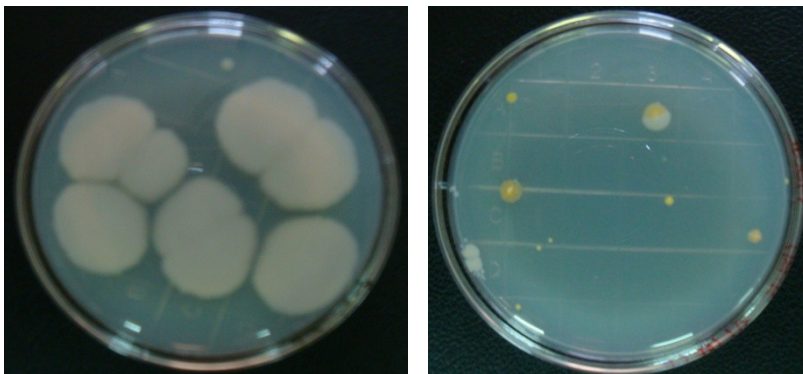
- *Results obtained from:*

- Benches : ELISA and Microscopes bench



Many UFC on ELISA bench and 4 on the microscopes bench. The limit is 5, so the results for the microscope bench are quite good while those for the ELISA bench are pretty bad. Keen interest should be taken in disinfecting the bench.

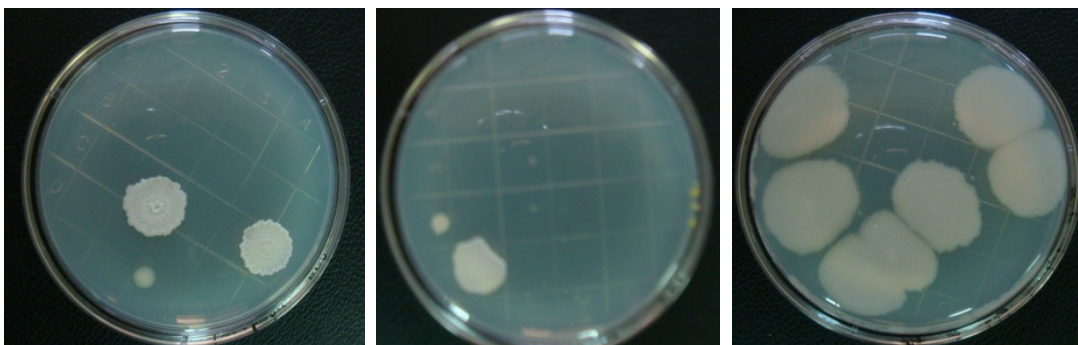
- Hoods



- 9 colonies on the big hood, 15 on the small hood. The results are pretty bad and this being the core area of aseptic manipulations, a great effort needs to be put to get meaningful results. Ensure proper disinfection before and after using the hoods.

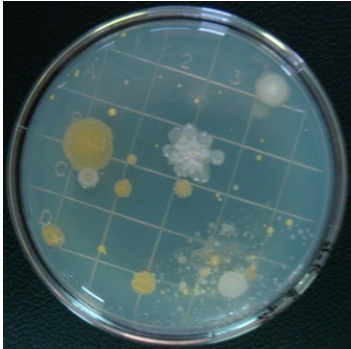
- Incubator 28°C and 37°C

Top of the rotative incubator

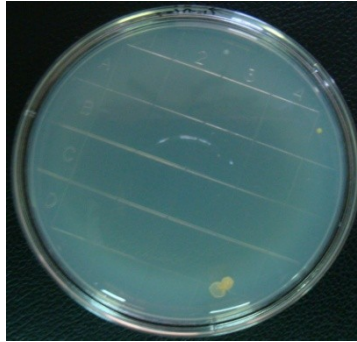


- 11 colonies in the 37°C incubator, 3 colonies in the 28°C. In addition to raising the temperature regularly, disinfection of the incubators can help us attain good standards.
- 7 colonies. This is above the limit and care should be taken to clean this surface regularly especially after use.

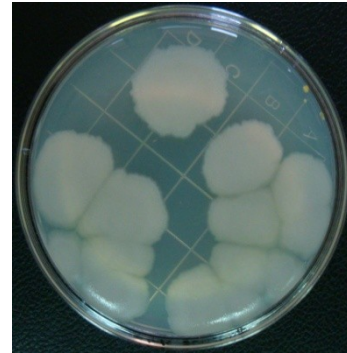
Top of the shelves



trolley



Sink surface



3 colonies. This is pretty good and should keep up this way.

More than 25 colonies. Caution should be taken for this surface and the general cleaning plan be followed to the latter.

15 colonies. This is not good. As we clean our hands and other apparatus, let us also make sure that this area is also left clean.

## 14.2 Conclusion

- Results are very bad this time round and show that we have completely forgotten the basics of using a lab i.e., proper cleaning and disinfection. This is a MICROBIOLOGY lab and if we cannot maintain the right standards then we cannot trust the results we get here. Let us put special efforts so that we can get better results than what we have now. We have had better results before so we can still improve.
- We need to be pay attention on the disinfection part and do it as per indicated in the cleaning plan.

Surfaces	Limits	Results	Conclusion
Bench ELISA	5 CFU/plate	>25 CFU/plate	Off limits, need to improved
Bench microscopes	5 CFU/plate	4 CFU/plate	Ok, but just at the limit
Big hood	2 CFU/plate	9 CFU/plate	Off limits, need to improved
Small hood	2 CFU/plate	15 CFU/plate	Off limits, need to improved
Top of the rotative incubator	5 CFU/plate	7 CFU/plate	Off limits, need to improved
Incubator 28	2 CFU/plate	3 CFU/plate	Off limits, need to improved
Incubator 37	2 CFU/plate	11 CFU/plate	Off limits, need to improved
Sink surface		15 CFU/plate	Off limits, need to improved
Trolley		3 CFU/plate	
Top of shelves		>25 CFU/plate	Off limits, need to improved

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