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DRAFT EAST AFRICAN STANDARD

Biofertilizers — Specifications

EAST AFRICAN COMMUNITY

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Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in the East African Community. It is envisaged that through harmonized standardization, trade barriers that are encountered when goods and services are exchanged within the Community will be removed.

In order to achieve this objective, the Community established an East African Standards Committee mandated to develop and issue East African Standards.

The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the private sectors and consumer organizations. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the procedures of the Community.

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

The committee responsible for this document is Technical Committee EASC/TC 020, *Agriculture and agrochemicals.*

Introduction

Biofertilizers are substances, which contain living microorganisms, which, when applied to seed, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of nutrients to the host plant.

Biofertilizers add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing phosphorus 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, lactic acid producing bacteria and zinc-solubilising biofertilizers. The use of biofertilizers offers economic and ecological benefits by way of soil health improvement and fertility.

Biofertilizers, known as microbial products, act as nutrient suppliers and soil conditioners that lower agricultural burden and conserve the environment. Good soil condition is imperative to increased crop production, as well as human and/or animal health welfare. Thus, the materials used to sustain good soil condition, are treated as environmental matters. However, there are still some problems to be met on the use of microbial products. More precise quality control should be made in favour of the customers. With this in mind, we will do our best to develop better production techniques and to improve the management system for microbial products.

Although the effects of biofertilizers are different among countries due to variances in climate and soil conditions, the importance of biofertilizers on environmental conservation in the 21st century should not be ignored. In the same manner, various acceptable biotechnologies can be used for increasing the biofertilizers effects with concern for the environment.

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

Biofertilizer — Specification

1 Scope

This draft East African standard specifies requirements, methods of sampling and test for biofertilizers. This draft standard covers the following types of biofertilizers; Rhizobia, Phosphate solubilizing microorganism, Azospirillum and Azotobacter.

2 Normative references

The following referenced documents are indispensable for the application of this standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8157, *Fertilizers, Soil Conditioners and beneficial substances – Vocabulary*

AOAC 2006.03, *Arsenic, cadmium, cobalt, chromium, mead, molybdenum, nickel, and selenium in fertilizers — Microwave digestion and inductively coupled plasma-optical emission spectrometry*

AOAC 965.08, Water (free) in fertilizers — Vacuum-desiccation

ISO 8397, Solid fertilizers and soil conditioners – Test sieving

ISO 4832, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique*

ISO 10390, *Soil, treated biowaste and sludge — Determination of* pH

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

ISO 5315, *Fertilizers — Determination of total nitrogen content — Titrimetric method after distillation*

ISO 15958, *Fertilizers — Extraction of water soluble phosphorus*

ISO 14820-1, *Fertilizers and liming materials — Sampling and sample preparation — Part 1: Sampling*

ISO 14820-2, Fertilizers and liming materials — Sampling and sample preparation — Part 2: Sample preparation

3 Terms and definitions

For the purposes of this standard, the terms and definitions given in ISO 8157 and the following shall apply.

3.1 biofertilizer

any substance which contains live microorganisms, part of microorganisms or product of microorganisms in their natural or modified forms that is manufactured, sold or represented for use in the improvement of the condition of soils or to aid plant growth or crop yield

3.2 efficacy

the ability of a fertilizer or supplement to fulfil any label claims and to produce a desired or intended result based on the labelled guarantees and directions for use

3.3 efficacy assessment

evaluate product performance in order to establish appropriate label claims, active ingredient guarantee and usage patterns

3.4 active agent

living agent in a biofertilizer to which the improvement of the condition of soils, nutrient availability or aid in plant growth or crop yield are attributed

3.5 inoculants

preparations containing beneficial micro-organisms in a viable state, intended for increasing the number of desired microorganisms.

3.6 bio fertilizer carriers

substances which support, present and preserve microorganisms in a viable state

3.7 biological nitrogen fixation

a process by which the atmospheric nitrogen gas is converted into forms that are easily absorbed by plants through biological processes.

3.8 nodules

swellings or outgrowths on roots and stems particularly of leguminous plants resulting from the activity of some atmospheric nitrogen fixing microorganisms.

3.9 plant growth promoters/enhancers/phytohormones

substances which stimulate the growth and development of plants

3.10 symbiotic microorganisms

microorganisms that form a mutual relationship with plants.

3.11 mineralization

biological breakdown of organic materials to release inorganic nutrients for plant uptake

3.12 immobilization

process whereby inorganic nutrients are locked up in microbial cells thus rendering them unavailable to plants

3.13 solubilization

a process, where bound nutrients are dissolved by microbial action.

3.14 substrate

any material that serves as source of nutrient for microorganisms.

3.15 Strain

specific variant or subpopulation of a microorganism that exhibits distinctive characteristics, often as result of genetic or environmental differences

4 Requirements

4.1 General requirements

4.1.1 Biofertilizer shall contain effective strain in minimum recommended population.

4.1.2 Biofertilizer shall not contain more than the maximum allowed contaminating microorganisms.

4.1.3 Biofertilizer shall not contain genetically modified microorganisms

4.1.4 Biofertilizer shall have single or a combination of effective strains.

4.1.5 Biofertilizers shall contain no pathogenic organisms which could affect plants, animals and human beings.

4.1.6 Biofertilizers carriers shall be of a nature that is not harmful to the environment

4.1.7 Biofertilizer shall have at least one of the following properties:

a) ability to fix nitrogen;

b) ability to solubilize minerals;

c) ability to improve plant performance.;

4.1.8

The use of biofertilizers shall take into consideration the effect of the applied inoculants against inherent beneficial microbiome.

4.2 Specific requirements

4.2.1

Bio fertilizer shall comply with the following requirements based on the declared active agent as stipulated in Table 1 - Table 4.

Table 1 — Specific requirements of rhizobia containing biofertilizer

SL/No.	Parameter	Requirement	Test Methods
i.	Base	Carrier; solid or liquid based	
ii.	Viable cell count (CFU) 15 days after manufacturing and 15 days before expiry date	Minimum $5x10^7$ cell/g of carrier material or 10^8 cell/mL of liquid	Annex E
iii.	Contaminating microorganisms level	No contamination at 10^5 dilution	Annex E
iv.	pH	6.5 - 7.5	ISO 10390
v.	Particle size(solid carrier-based)	All material shall pass through 0.15-0.212 mm IS sieve	ISO 8397
vi.	Moisture, % by weight, max. (solid carrier-based)	30% - 40%	AOAC 965.08
vii.	Nodulation test	Should be positive	Annex F
viii.	Efficiency character	Should show effective nodulation on all species (crops) listed on the packet nodulation (should appear pink when dissected)	Annex F
ix.	Shelf life	Should have at least 6 months for solid carrier-based inoculants and 6 weeks for liquid-based inoculants (under refrigeration) from date of manufacturing	

Table 2 — Specific requirements of phosphate solubilizing microorganisms containing biofertilizers

SL/No.	Parameter	Requirement	Test Methods
i.	Base	Carrier; solid or liquid based	
ii.	Viable cell count (CFU) 15 days after manufacturing and 15 days before expiry date	Minimum $5x10^7$ cell/g of carrier material or 10^8cell/ mL of liquid	Annex G
iii.	Contaminating microorganisms level	No contamination at 10^5 serial dilution	Annex G
iv.	pH	6.5 - 7.5 (for solid) 5 - 7.5 (for liquid)	ISO 10390
v.	Particle size (solid carrier-based)	All material shall pass through 0.15-0.212 mm IS sieve	ISO 8397
vi.	Moisture, % by weight, max. (solid carrier-based)	35% - 40 %	AOAC 965.08
vii.	P –solubilization	30% - 50 %	Annex H
viii.	P- solubilisation zone, min	1 mm	Annex G
ix.	Shelf life	Should have at least 6 months for solid carrier-based inoculants and 6 weeks for liquid-based inoculants (under refrigeration) from date of manufacturing	

Table 3 — Specific requirements of azospirillum containing biofertilizer

SL/No.	Parameter	Requirement	Test Methods
i.	Base	Carrier; solid or liquid based	
ii.	Viable cell count (CFU) 15 days after manufacturing and 15 days before expiry date	Minimum $5x10^7$ cell/g of solid carrier material or10^8cell/mL of liquid	Annex I
iii.	Contaminating microorganisms level	No contamination at 10^5 serial dilution	Annex I
iv.	pH	7.0 – 8.0	ISO 10390
v.	Particle size(carrier-based)	All material shall pass through 0.15-0.212 mm IS sieve	ISO 8397
vi.	Moisture, % by weight, max. (carrier-based)	35% - 40 %	AOAC 965.08
vii.	Efficiency Character	Formation of white pellicle in semi solid nitrogen free bromothymol blue media. 1. Shall show effective root development on all cultivar/crops	Annex J
viii.	Shelf life	Should have at least 6 months for solid carrier-based inoculants and 6 weeks for liquid-based inoculants (under refrigeration) from date of manufacturing	
ix.	P- solubilisation zone	1 mm	Annex G

Table 4 — Specific requirements of Azotobacter containing biofertilizer

SL/No.	Parameter	Requirement	Test methods
i.	Base	Carrier; solid or liquid based	
ii.	Viable cell count (CFU) 15 days after manufacturing and 15 days before expiry date	Minimum $5x10^7$ cell/g of solid carrier material or10^8cell/mL of liquid	Annex K
iii.	Contaminating microorganisms level	No contamination at 10^5 dilution.	Annex K
iv.	pH	6.5 – 7.5	ISO 10390
v.	Particle size(carrier-based)	All material shall pass through 0.15-0.212 mm IS sieve	ISO 8397
vi.	Moisture, % by weight, max. (solid carrier-based)	30% - 40 %	AOAC 965.08
vii.	N-fixation	Not less than 10 mg N/g of sucrose	Annex L
viii.	Shelf life	Should have at least 6 months for solid carrier-based inoculants and 6 weeks for liquid-based inoculants (under refrigeration) from date of manufacturing	

5 Contaminants

Heavy metal contamination in Bio-fertilizers shall not exceed the limits given in table 5 when determined by the methods prescribed in AOAC 2006.03.

Table 5 — Heavy metal contaminants limits

SL No.	Parameter	Limits in ppm	Test methods
i)	Arsenic, As, max.	10.0	AOAC 2006.03
ii)	Cadmium, Cd, max.	5.0	
iii)	Mercury, Hg, max.	0.1	
iv)	Chromium, Cr, max.	50.0	
v)	Lead, Pb, max.	30.0	

6 Sampling

6.1 Scale of sampling

6.1.1 Lot

All containers in a consignment belonging to the same batch of manufacture shall constitute a lot. If the consignment is declared to consist of different batches of manufacture, containers of the same batch shall be grouped together and each group so formed shall constitute a separate lot.

Sample shall be tested from each lot for ascertaining conformity to the requirements of this standard.

6.1.2 Sample size

The number of containers to be selected from a lot for testing for microbiological and other requirements shall depend on the size of the lot and shall be in accordance with Table 6.

Table 6 – Number of containers to be selected for sampling

No. of containers in the lot	No. of containers to be selected (n)	
	Microbiological	Other tests
up to 1300	12	18
1301 to 3200	18	24
3201 and above	24	30

6.1.3 Sampling method

The containers to be selected for testing shall be chosen at random from the lot by the following procedure. Starting from any container, count them as 1,2,3….. up to r. Every r^{th} containers thus counted shall be withdrawn, r being the integral part of N/n, where N is the total number of containers in the lot and n is the total number of containers to be chosen (Table 6).

6.2 Test samples and reference samples

6.2.1 Samples for microbiological tests

The sample containers selected for microbiological tests (see col. 2 of Table 6) shall be divided at random into three equal sets and labelled with all particulars of sampling. One of these sets of sample containers shall be for the buyer; another for the supplier and the third set is the reference. The sample for testing should be handled as per prescribed conditions on the manufacturer label.

6.2.2 Samples for other tests

The sample containers selected for other tests (see col. 3 of Table 6) shall be divided at random into three equal sets and labelled with all the particulars of the sample. One of these sets of sample containers shall be for the buyer, another for the supplier and third is the reference.

6.2.3 Reference samples

Reference samples shall consist of set of sample containers for microbiological tests (see 6.2.1) and a set of sample containers for other tests (see 6.2.2) and shall bear the seals of the buyer and supplier or as agreed to between the two.

7 Packaging

Biofertilizers shall be packaged in the materials that are clean and non-defective that protects the product from physical, chemical and moisture contamination and withstand multiple stages of handling (transportation and storage).

8. Labelling

The following information shall be clearly labelled on the package legibly and indelibly in either Kiswahili or Kiswahili and English;

a) name of the product i.e. “Biofertilizer”

b) active ingredient shall appear in close proximity to the name of the product by specifying the genus and species of the microorganism;

c) brand name;

d) microbial density;

e) name and address of manufacturer, exporter, packer and/or dispatcher;

f) type/nature of carrier;

g) batch or code number;

h) storage conditions and instructions;

i) date of manufacture;

j) expiry date;

k) net content by weight;

l) product registration number;

m) directions/instructions for use including crops and how to apply

n) safety precautions;

o) Directions on Disposal

p) Compatibility

q) Declaration on GMO status

Annex A

(informative)

Procedures for quality control of biofertilizer

A1 Rhizobium

Quality checks on rhizobium biofertilizer can be divided into three parts:

a) Mother culture test

b) Broth test

c) Peat test

A1.1 Mother culture test

Before producing rhizobium bio fertilizer, the mother culture should be checked on the following:

a) Growth

b) Purity

c) Gram strain

a) Growth

By streaking a mother culture on yeast mannitol+Congo red agar (YMA) plates, checking the growth of rhizobia.

Fast-Growing rhizobia colonies will appear in 3-5 days and slow-growing rhizobia will appear in 5-7 days.

b) Purity

Check purity by streaking culture on glucose peptone agar plate, and incubate for 24 hours at 30 ºC. No growth or poor growth should be obtained on GPA. Good growth and colour changes can be expected from contaminants.

c) Gram stain

A lot of mother culture is checked by gram staining. Rhizobial cell is Gram-negative, retains safranin colour. Cells should appear red and not violet when observed under the microscope.

A1.2 Broth test

The following qualities of the broth samples shall be checked to make sure that the broths are in good condition.

a) pH

b) Staining

c) Optical density

d) Total count

e) Viable number

a) **pH**

Slow-growing rhizobia such as rhizobia for soybeans, mungbean and peanut produce a little basic compound.

After incubation, the pH will increase (example, pH before growing = 6.0. after growing pH = 6.1-6.2) If broth pH decreases, it means some contaminants occur; lower pH indicates presence of contaminants.

b) **Staining (Gram stain or fushin stain)**

Rhizobial cells are stained for observation of shape and size of the cells. Cells of rhizobia are rod-shaped, with one or two cells sticking together. They do not appear in long-chain. Long-chained cells are indicative of contaminants.

Gram- stained cells should appear red, not violet. Fuchsin staining is an easier and faster method. Rhizobial cells can be routinely checked using fuscshin stain.

Table 1 — Gram stain

Solutions		**Reactions and appearance of bacteria**	
		Gram-positive	**Gram-negative**
I	Crystal violet (CV)	Cells stain violet	Cells stain violet
II	Iodine solution	CV-I formed within cells; Cells remain violet	CV-I formed within cells; Cells remain violet
III	Alcohol	Cell walls dehydrated, Shrinkage of pores occurs, Permeability decreases, CV-I complex cannot pass out of cells, cells remain violet	Lipoid extracted from cell walls, porosity increases, CV-I is removed from cell
IV	Safranin	Cells not affected, remain violet	Cells take up this stain, become red.

c) **Optical density**

Broth culture with active rhizobial growth will become turbid in 3-4 days. Broth turbidity, or optical density using spectrophotometer (at 540 nm) will show readings of 0-1.0 O.D. The value of O.D correlates to number of cells. If values are high, then cells numbers are also high.

d) **Total count**

Total count includes viable cells and dead cells by using petroft-Hauser counter. At least 10 small squares all around the total area are counted, and not only in one large square.

Precautions:

a) cells have to be homogenous;

b) clumping of cells (use non-ionic detergent);

c) it gives total count only;

d) Petroft, cover slip shall be properly positioned to get uniform depth.

e) **Viable count (To check if its only informative or test method)**

The number of living cells is counted by spread plate or drop plate methods. Doing spread plate by making serial dilutions from 10^{-1} -10^{-6} or 10^{-7} (depend on concentration) then three replicates of 0.1 mL of broth from 10^{-6} and 10^{-5} are spread over the YMA+CR plates. Plates are incubated (at 28 ºC for seven days. Colonies of rhizobial cells are round, opaque and have smooth margin. They are white and do not absorb red colour as well as other bacteria. Calculation of the number of rhizobia per mL:

$$No.\ of\ cells\ /mL = \frac{No.\ of\ colonies \times dilution\ factor}{Volume\ of\ inoculum}$$

A1.3 Peat test

For the peat inoculants, these are the qualities to be checked:

a) pH

b) Moisture content

c) Viable number

d) Plant infection method (MPN)

a) **pH (check if it a test method or an informative)**

Maintain neutral pH for the inoculant. Since peat is acidic the pH is to be increased with CaCO3.Weigh 10 g of inoculant, pour 20 ml of distilled water, mix well with glass rod, incubate at least 30 minutes, and then measure with pH meter.

b) **Moisture content**

The optimum moisture content of peat-inoculant is between 40 %- 50 %. At low moisture rhizobia will die rapidly. If moisture is high, inoculants may stick to the plastic bag and, thus, not good for rhizobial growth.

c) **Viable number**

The number of viable rhizobia is counted by spread-plate method as in the broth test. It is more difficult when analyzing non-sterile peat. Colonies may sometimes be contaminated by other bacteria. Good expertise is required to conduct this microbiological analysis.

d) **Plant infection method**

Principle

This is an indirect method of assessing plant infection on nodulation. It is widely used when peat is not sterile. It takes more time than spread plate method (because plants have to be grown). MPN is usually done to compare the results with a spread plate method.

Assumptions

i) If a viable rhizobium is inoculated on its specific host, nodules will develop on those roots.

ii) Nodulation on that inoculated plant is a proof of the presence of infective rhizobia.

iii) Absence of nodule is a proof of the absence of infective rhizobia.

iv) Uninoculated plants are used as control, with absence of nodule.

Conditions

i) Nodulation on that inoculated plant is a proof of the presence of infective rhizobia. (Nodules however shall have pink nodules if cut open hence showing effective bacteria present).

ii) Absence of nodule is a proof of the absence of infective rhizobia or presence of white (meaning noninfective) or grey/green (dead) bacteria in the nodules.

Estimation of MPN

Plants within any given pouch are considered as a growth unit. Nodulation is recorded positive for 'nodulated growth unit' or negative for absence of nodule. The actual number of nodules on each plant has no meaning on MPN count. If replications are in quadruplicated, the reading may be 4,3,2,1 or 0 units. The highest dilution should show no nodulation.

The estimated number rhizobium per g is calculated by the formula:

$$X = \frac{m * d}{V}$$

Where

M is the number from MPN,

d is the lowest dilution (first unit),

V is the volume of aliquot inoculated.

Contaminants have some effect on counting. In the presence of contaminants; count of MPN will give lower results than plate counts.

A2 Non- symbiotic nitrogen fixer

In the laboratory, microbial growth may be represented by increase in cell mass, cell number or any cell constituent. Utilization of nutrients or increase in metabolic products can also be related to the growth of the organism.

Growth, therefore, can be determined by several techniques based on one of the following measurements:

a) Cell count directly by microscopy or by an electronic particle counter, or indirectly by colony count.

b) Cell mass, directly by weighing or measurement of cell nitrogen, or indirectly by turbidity.

c) Cell activity, indirectly by relating the degree of bio chemical activity to the size of the population.

A2.1 Procedure for bacterial cells count by hemocytometer

If the formulation is tested only for bacterial cell counting which will not form colonies on agar medium, it can be tested directly in hemocytometer by adopting the Standard Serial Dilution Method. The total bacterial cells can be observed under microscope

A2.1.1 Requirements

Sample 0.1 mL (Bacterial suspension - either from activated sample or directly from the formulation) and hemocytometer (Counting chamber)

A2.1.2 Procedure

Place a drop of product suspension made from liquid culture (Filtered through 1 or more layer of muslin cloth) on the engraved grid and let the preparation stand for 1 to 2 minutes to allow the bacterial cells to settle at the bottom.

Put the cover glass over the grid carefully so that no air bubble enters between the slide and cover glass.

Slide the cover glass backwards and forwards until colored rings are visible as the two surfaces of the cover glass and slide into close contact.

Count the bacterial cells in the middle square (E), which consists of 25 groups of 16 small squares, each group 0.2 mm squares.

A2.1.3 Results

Calculate the number of spores per mL of the suspension mathematically as follows:

No of cells per mL of the product = $\frac{D * X}{N * K}$

Where,

D is the dilution factor

X is the total number of polyhedral counted

N is the Number of Squares counted

K is the Volume above one small squares in cm3

A3 Mycorrhiza-the Arbuscular micorrhizal fungi, (AMF)

Quality control in the production of AMF inoculums is essential for product consistency, reliability and reproducibility. This is applied to the labor atory, preparation room, growth room, and storage room and green houses, taking care into the design to achieve the most efficient control in inoculums production.

A3.1 Laboratory quality control

i) Spores are extracted from selected batches of mono specific spore cultures in the preparation room.

ii) The spores are transported in Petri dishes to the laboratory and placed in the refrigerator before examination.

iii) The Petri dishes are examined under stereoscopic microscopes.

iv) Description of the spores from each Petri dish is recorded.

v) Petri dishes are then cleaned and dried.

A3.2 Preparation room quality control

i) This room has to be isolated from the green house and growth room and should not receive unsterilized

soil or potting media samples

ii) Stored materials (cultures; sterilized growth media) are clearly labelled and placed in specific containers

iii) Floor should always be clean, avoiding sweeping which encourages distribution of dust

iv) Benches and other surfaces are cleaned with wet towels.

v) Containers are surface-sterilized with 10% sodium hypochlorite.

A3.3 Growth room quality control

i) The growth room should be temperature controlled (22 ºC) and air is exhausted to the outside (no recycling of stale air);

ii) Bench tops should be painted with anti-microbial paint;

iii) All surfaces should be sterilized periodically e.g. monthly;

iv) All samples are checked for contaminants and pathogens;

v) Watering is done manually with great care to avoid cross-contamination.

A3.4 Storage room quality control

i) All samples stored are placed in plastic bags, with proper labelling and surface of bags should be wiped clean before storage.

ii) Floors and bench tops are wiped regularly, and dusting or sweeping should be avoided to prevent generation of dust.

A3.5 Most probable number (MPN) — Method for estimating number of VAM fungi infective propagules in VAM products

A3.5.1 Introduction

The MPN technique gives a more realistic estimate of the number of infective propagules of VAM fungi in VAM products. It is very useful for VAM fungi because

i) viable propagules include spores and hyphae (soil, roots),

ii) spores of some VAM fungi may be difficult to extract,

iii) some VAM fungi produce only a few infective spores, and

iv) VAM fungi could complete their life-cycle without sporulating.

The major advantage of MPN technique is that it enumerates all propagules which have the ability to infect at a given point of time.

A3.5.2 Requirements

Twenty pots (70 cm3), 40 presoacked seeds, sterilized sand: soil (1:1) mixed soil dilutions, polythene bags (30 cm x 20 cm) and physical balance.

A.3.5.3 Procedure

Take 30 g of test VAM product in a polythene bag. Add 270 g of sterilized sand: soil (1:1) mix. Shake

thoroughly (50 - 75 shakes) to get 10-1 dilution.

Make a ten-fold series of VAM product dilutions with the test soil up to 10-1 dilution (go for higher dilution if needed)

Place diluted VAM product in pots (5 pots per dilution)

Two seeds of selected host are sown in each pot

Allow the plants to grow in the polyhouse or growth chamber for 30 days after germination.

Wash, clear and stain the entire root systems with trypan blue and examine microscopically. Record the presence or absence of colonization for each root system separately.

For each of the 5 replicates in each of the four dilutions (100, 10-1, 10-2 and 10-3), one might obtain a combination of numbers as shown below:

Dilutions	0	1/10	1/100	1/1000
No. of plants colonized	5	5	3	2

This means that all the 5 replicate pots are positive for VAM colonization in dilutions 10^{-1} and 10^{-2}, three are positive pots in dilution 10^{-3} and 2 positive pots in dilution 10^{-4}. For the calculation of MPN of propagules only three numbers of the given combination are required as follows:

N_1	N_2	N_3
10^{-1}	10^{-2}	10^{-3}
5	3	2

The first number (N1) is that corresponding to least concentrated dilution in which all (or the greatest number of) the tubes are positive for AM colonization. The two other numbers (N2 and N3) are those corresponding to the next two higher dilutions.

The most probable number of AM propagules can then be calculated using MPN Table. Making use of the table with the values of N1, N2 and N3, the value given for the combination 5 3 2 is 1.4. This is the MPN Index value.

To obtain the MPN of infective propagules of VAM fungi in the sample MPN Index value has to be multiplied by the middle dilution factor ie. 1.4 x 10^3 = 140 propagules cm3 of VAM product.

A3.6 VAM fungi root infectivity test

Roots are removed from the soil and washed gently with tap water and then collected on a 0.5-mm mesh that removes soil particles and retains the small root fragments in order to determine the mycorrhizal status of each species in different plots. The roots are fixed in FAA (formalin, glacial acetic acid, 95 % ethyl alcohol, and water 2: l:10:7 v/v/v/v), digested at 90 ºC for 1 h. in 7 % KOH solution, and stained in 0.05% trypan blue (500 mL glycerol, 50mL 1% HCl, 0.5 g trypan blue, and 450-mL water) for 10-15 min at 90 ºC; this is a slight modification of the Phillips and Hayman (1970) method. After staining, excess stain is removed, and the roots are destained in clear acidified glycerine (500 mL glycerol, 450 mL water, and 50 mL 1% HCl). Roots are washed and cut into 1-cm segments. 30 to 40 root segments are randomly selected, placed on a glass slide, covered with a cover slip, and examined with a microscope to detect the presence or absence of colonization by VAM fungi. Only those segments containing mycorrhizal hyphae and either vesicles or arbuscules are counted as colonized. The number of root segment colonized is expressed as a percentage of total root segments in the sample.

Annex B

(informative)

Biofertilizers medium composition

B.1 Rhizobium

- Medium composition
- K_2HPO_4 - 0.5 g
- $MgSO_4,7H_2O$ - 0.2 g
- NaCl - 0.1 g
- Mannitol - 10.0 g
- Yeast extract - 1.0 g
- Agar - 15.0 g
- Distilled water - 1 litre
- 1 per cent aqueous Congo red 2.5 mL
- Adjust the pH at 7 ± 0.2 with N/10 HCl and N/10 NaCl
- Autoclave at 15 psi pressure and 121°C for 30 min

B.2 Azotobacter

Medium Composition - (Jensen's medium)

- Sucrose - 20.0 g
- K_2HPO_4 - 1.0 g
- $MgSO_4.7H_2O$ - 0.5 g
- NaCl - 0.5 g
- $FeSO_4$ - 0.1 g
- $CaCO_3$ - 2.0 g
- Agar - 15.0 g
- Distilled water - 1 litre
- Adjust the pH at 7.
- Autoclave at 15 psi and 121°C for 30 minutes.

B.3 Azospirillum:

Medium composition (Okon's medium)

- K_2HPO_4 - 6.0 g
- K2HPO4 - 4.0 g
- Distilled water - 500 mL
- $MgSO_4$ - 0.2 g
- NaCl - 0.1 g
- $CaCl_2$ - 0.02 g
- NH_4Cl - 1.0 g
- Malic acid - 5.0 g
- NaOH - 3.0 g
- Yeast extract - 0.05 g
- Na2MoO4 - 0.002 g
- MnSO4 - 0.0001 g
- H3BO3 - 0.0014 g
- Cu(NO3)2 - 0.0004 g
- ZnSO4 - 0.0021 g
- FeCl3 - 0.002 g
- Distilled water - 500 mL
- Bromothymol blue - 2 mL

- (0.5 per cent alcoholic solution)

B.4 Phosphobacter

Medium composition (Pikovskaya's medium)

- Glucose - 10.0 g
- Ca3(PO4)2 - 5.0 g
- (NH4)2SO4 - 0.5 g
- KCl - 0.2 g
- MgSO4.7H2O - 0.1 g
- MnSO4 - trace
- FeSO4 - trace
- Yeast extract - 0.5 g
- Agar - 15.0 g
- Distilled water - 1 litre
- Sterilize the medium at 10 psi pressure for 30 minutes

Annex C

(informative)

Properties of a good carrier material for seed inoculation

a) non-toxic to inoculants bacteria strain

b) good moisture absorption capacity

c) easy to process and free of lump-forming materials

d) easy to sterilize by autoclaving or gamma-irradiation.

e) available in adequate amounts

f) inexpensive

g) good adhesion to seeds

h) good pH buffering capacity

i) non-toxic to plant

Annex D

(informative)

Common carriers for biofertilizers

SL/NO	Carrier material	Inoculum bacterium	Characteristic
i.	Sterilized oxalic acid industrial waste	Rhizobium	-seed inoculation -Rhizobium multiplication in carrier in ambient temperature up to 90 days.
ii.	Alginate-perlite dry granule	Rhizobium	-soil inoculation -the inoculants can be stored in a dry state without losing much viability.
iii.	Composted sawdust	Bradyrhizobium, rhizobium and Azospirillum	-seed inoculation -good growth and survival of inoculants strains
iv.	Mineral soils	Rhizobium	-seed inoculant
v.	coal	Rhizobium	-seed inoculant
vi.	Soybean oil or peanut oil added with lyophilized cells	Rhizobium	-seed inoculant
vii.	Wheat bran, sugar cane baggers	Rhizobium/brady rhizobium and rock phosphate solubilising fungus *Aspergillus niger*	-soil inoculant
viii.	Nutrient supplemented pumice	Rhizobium	-seed inoculants

Annex E
(normative)

Determination of number of Rhizobium cells

Reagents

C.1.1 **Congo Red** – 1% of aqueous solution

C.1.2 **Preparation of culture medium**: Yeast extract mannitol broth

a) Use a plating medium of the following composition:

Agar	20 g
Yeast extract	1 g
Mannitol	10 g
Potassium hydrogen phosphate (K_2HPO_4)	0.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2 g
Sodium Chloride (NaCl)	0.1 g
Congo red	2.5 ml
Distilled water	1000 ml
Ph	7.0

C.1.3 The culture medium shall be prepared in accordance with ISO 11133 or using any other acceptable validated test method.

Preparation of serial dilution for plate count

C.2.1 Dispense 30 g of Inoculants to 300 ml of sterile distilled or demineralized water and shake for 10 min on a reciprocal shaker or homogenizer.

C.2.2 Make serial dilutions up to 10^{-10} by suspending 10 ml aliquot of previous dilution to 90 ml of water.

C.2.3 Take 0.2 ml or suitable aliquot of 10^{-6} to 10^{-8} dilutions using sterile pipettes and deliver to petri dishes containing set medium as given in C.1.2 and spread it uniformly with a spreader. Invert the plates and promptly place them in the incubator.

Incubation of plates

Label the plates and incubate at 28 ± 2 °C for 3 to 5 days for fast growing Rhizobia and 5 to 10 days for slow-growing ones.

Colony counting aids

Count the colonies with the aid of magnifying lens under uniform and properly controlled, artificial illumination. Use a colony counter, equipped with a guide plate and rules in Centimeter Square. Record the total number of colonies with the hand tally. Avoid mistaking particles of undissolved medium or precipitated matter, in plates for pinpoint colonies. To distinguish colonies from dirt, specks and other foreign matter, examine doubtful objects carefully.

Colony count

C.5.1 Count all plates but consider for the purpose of calculation plates showing more than 30 and less than 300 colonies per plate. Disregard colonies, which absorb Congo red and stand out as reddish colonies.

C.5.2 *Rhizobium* forms white, translucent, glistening, elevated and comparatively small colonies on this medium. Moreover, *Rhizobium* colonies do not take up the colour of congo red dye added in the medium. Those colonies which readily take up the congo red stain are not rhizobia.

C.5.3 Count such colony numbers and calculate figures in terms of per gram, of carrier. Also check for freedom from contamination at 10^{-8} dilution.

Annex F (normative)
Test for nodulation (Pot culture test)

Plant nutrient solution

Reagents

	Composition	Concentration	g/l
(1)	Potassium chloride	0.001M	0.0745
(2)	Di-Potassium hydrogen Phosphate (K_2HPO_4)	0.001 M	0.175
(3)	Calcium sulphate ($CaSO_4.2H_2O$)	0.002 M	0.344
(4)	Magnesium sulphate ($MgSO_4.7H_2O$)	0.001 M	0.246
(5)	Trace elements solution:		0.5 ml
	1. Copper sulphate ($CuSO_4$ $5H_2O$)	0.01mg/kg	0.78
	2. Zinc Sulphate ($ZnSO_4$ $7H_2O$)	0.25 mg/kg	2.22
	3. Ammonium molybdate ($(NH_4).6Mo7O_24.4H_2O$)	0.0025 mg/kg	0.01
	4. Magnesium sulphate ($MgSO_4.7H_2O$)	0.25 mg/kg	2.03
	5. Boric acid (H_3BO_4)	0.125 mg/kg	1.43
	6. Water		1000 ml
	Prepare the solution no (5) consisting of trace elements in one litre of stock solution and add to final nutrient solution at the rate of 0.5 ml per litre.		
(6)	Iron solution		0.5 ml
	1. Ferrous sulphate		5
	2. Citric acid		5
	3. Water		100 ml
	Prepare the solution no. (6) As 100 ml of stock solution and add final nutrient solution at the rate of 0.5 ml per litre.		

Preparation of plant nutrient solution

D.1.2.1 Prepare the nutrient solution by weighing out substances (1), (2) and (4) and dissolving them in a liter of water.

D.1.2.2 To this solution add 0.5 ml of trace elements solution and 0.5 ml of iron solution.

D.1.2.3 Grind in a mortar 0.344 g of calcium sulphate (3) to a fine consistency and add to the final nutrient solution.

D.1.2.4 Autoclave the nutrient solution thus prepared, at 121 °C for 15 min.

NOTE 1 The nutrient solution is prepared in the tap water provided the water is soft.

NOTE 2 The nutrient solution has to be shaken well to disperse calcium sulphate before dispensing.

NOTE 3 If the solution is made up with distilled water, the pH is about 7.2 before autoclaving and falls to 5.5 on autoclaving and rises slowly on standing to about 5.8. However, there is no need to adjust pH. For most tropical legumes; pH of about 6.0 is adequate.

Procedure for nodulation

D.2.1 Immerse the seeds in 95 percent alcohol and follow by surface sterilization in freshly prepared chlorine water (for 15 to 20 min) or 0.1 percent mercuric chloride solution 3 min in a suitable container such as a screw-capped bottle or a test tube with a rubber hung.

NOTE 1 In case of seeds with tough seed coat, concentrated sulphuric acid has tobe used as a surface Sterilants for 20 to 30 min.

NOTE 2 It is recommended that the seeds are placed overnight in a desiccators containing calcium chloride before surface sterilization with sulphuric acid.

D.2.2 Pour out the Sterilants and wash the seeds in several changes of sterile water and wash the seeds in several changes of sterile water (at least ten times) to get rid of the Sterilants.

D.2.3 Fill earthenware or glazed pot with soil (2 parts soil and 1 part washed coarse sand) (pH 6 to 7) and autoclave for 2 h at 120 °C.

D.2.4 After two-days incubation at room temperature, repeat autoclaving to ensure complete sterility of soil. Inoculate surface sterilized seeds with water slurry of the inoculants taken from a culture packet (15 to 100 g seeds per gram of inoculants depending on the size of the seed) and sow the seeds. Keep a set of pots with uninoculated seeds as control and also a set of pots with ammonium nitrate at the rate of 100 kgN/ha as control aid incubate them in a pot-culture house during appropriate seasons for appropriate plants, taking care to separate the inoculated pots from the control pots.

NOTE If growth rooms or cabinets having facilities to adjust temperature and light are available, the pots are incubated in such controlled environmental conditions.

D.2.5 Sterilize the nutrient solution at 120 °C for 20 min and irrigate each pot once to the moisture holding capacity of soil. Subsequently, water the seedling periodically with sterilized water preferably through a plastic tube, taking care to prevent splashing of water from inoculated pots to uninoculated ones.

D.2.6 Maintain required number of replicated pots (4 to 6) for each botanical species for statistical analysis.

D.2.7 After two to three weeks of growth, thin down the number of plants in each pot to four uniform plants.

D.2.8 At the end of 6 to 8 weeks, take one set of pots from both the control and inoculated series and, separate the plants carefully from the soil under slow running water. Obtain data on the number, colour (effective nodules are pink or red) and mass of nodules.

D.2.9 At the end of 6 to 8 weeks, harvest the shoot system, dry at 60 °C for 48 h and determine dry mass. For the above purpose, maintain adequate replications of pots (4 to 16).

D.2.10 Record the nodulation data regarding formation of pink colour of nodules as revealed visually when nodules are cut open by razor blade. After computing the data, based on the dry mass of plants and nodulation data decide the effectiveness of culture. If good effective pink nodulation is obtained in inoculated plants together with local absence or sometimes presence of stray nodules in controls and if there is a 50% increase in the dry mass of plants over the uninoculated control without nitrate, it may be concluded that the culture is of the required quality.

Annex G
(normative)

Determination of number of phosphate solubilizing bacterial cells

Preparation of culture medium

A.1.1 Use a medium of the following composition:

Glucose	10.0 g
Tri-calcium phosphate	5.0 g
Ammonium sulphate	0.5 g
Magnesium sulphate	0.1 g
Sodium chloride	0.2 g
Yeast extract	0.5 g
Manganese sulphate	Trace
Ferrous sulphate	Trace
Distilled water	1000 ml
Agar	15.0 g
pH	adjusted to 7 ± 0.2

A.1.2 The culture medium shall be prepared in accordance with ISO 11133 or using any other acceptable validated test method.

Preparation of serial dilution for cell counts

A.2.1 Dispense 30 g of PSBI in 270 ml of sterile water and shake for 10 minutes on a reciprocal shaker.

A.2.2 Make serial dilutions up to 10^{-7} level.

A.2.3 Pipette out 0.2 ml aliquots of 10^{-5} to 10^{-7} dilution and deliver it on the Petri dishes containing set medium as described in A.1.

A.2.4 Spread the aliquots over the plate. Invert the plates and place them in the incubator at 28 ± 2 °C for 3 days.

A.2.5 Use 3 replicates of 10^{-5}, 10^{-6} and 10^{-7} dilution.

Counting

Count the total number of colonies on the plates including colonies with solubilization zone with the help of a colony counter.

Methods for counting solubilisation zones

A.4.1 Take 10 g of PSBI (BF) in 90 ml in water.

A.4.2 Make a tenfold dilution series up to 10^{-7}

A.4.3 Take 0.2 ml aliquots of 10^{-5} to 10^{-7} dilution using sterile pipettes and delivered to Petri dishes containing Pikovskayei's media.

A.4.4 Spread it uniformly. Invert the plate and incubate them up to 2 weeks at 28 ± 2 °C.

A.4.5 Count the colonies showing hallow cones and measure their diameter. Minimum acceptable zone is 10 mm in diameter.

Annex H
(normative)

Determination of soluble phosphorus using ascorbic acid

Principles

C.1.1 Soluble phosphorus forms hetropoly molybdo-phosphate complex with molybdate ions which on reduction produces a characteristic blue colour measured at 840 to 880 nm.

C.1.2 Considering the higher stability of the ascorbic acid, easiness to handle, higher tolerance to the concentration of interfering ions, possibilities to use it with all types of acids and higher stability the developed colour (from 10 to 60 min), ascorbic acid instead of stanous chloride is nowadays use as the reducing agent for the hetropoly molybdo-phosphate complex formed by the soluble phosphate ions on addition of ammonium molybdate solution.

Apparatus

Spectrophotometer capable of transmission measurements at 840 to 880 nm. Extractant: It is olsen extract.

Reagents

C.3.1 Ammonium molybdate $[(NH_4)_6M0_7O_{24}.4H_2O]$

C.3.2 L-Ascorbic Acid

C.3.3 p-Nitrophenol

C.3.4 10 N H_2S0_4

C.3.5 4 N H_2S0_4

Preparation of reagents

Sulphomulybdic acid

C.4.1.1 Take 20 g of ammonium molybdate and dissolve in 300 ml of distilled water.

C.4.1.2 Add slowly 450 ml of 10 N H_2SO_4.

C.4.1.3 Cool the above mixture and add 100 ml of 0.5 percent solution of antimony potassium tartrate.

C.4.1.4 Cool and make the volume to one litre.

C.4.1.5 Store in glass bottle away from direct sunlight.

Preparation of mixed reagents

C.4.2.1 Add 1.5 g of *L-ascorbic* acid in 100 ml of the above stock solution and mix. Add 5 ml of this solution to develop colour.

C.4.2.2 Mixed reagent is to be prepared fresh as it does not keep for more than 24 h.

Procedure

C.4.3.1 Weigh the required material in a 100 ml conical flask.

C.4.3.2 Add 50 ml of extractant and shake it of 30 min on a rotary shaker.

C.4.3.3 Filter the suspension through Whatman filter paper No. 40. If the filtrate is coloured then add a tea spoon of Darco-60 (activated phosphorus free carbon), shake and filter.

C.4.3.4 Take a known aliquot (5 to 25 ml) of the extract in a 50 ml volumetric flask.

C.4.3.5 Add 5 drops of p-nitrophenol indicator (1.5 percent solution in water) and adjust the pH of the extract between 2 and 3 with the help of $4NH_2SO_4$. The yellow colour will disappear when the pH of the solution becomes 3. Swirl gently to avoid loss of the solution along with the evolution of CO4.(CO_2.)

C.4.3.6 When the CO_2 evolution has subsided, wash down the neck of the flask and dilute the solution to about 40 ml.

C.4.3.7 Add 5 ml of the sulphomolybdic acid mixed reagent containing ascorbic acid. Swirl the content and make up the volume.

C.4.3.8 Measure the transmission after 30 min at 880 nm using red filter. The blue colour developed remains stable up to 60 minutes.

C.4.3.9 Record the concentration of phosphorus (P) in the extract from the standard curve.

Calculation

Calculate the concentration of soluble Phosphorus using the following formula:

$$P\ (\%) = \frac{z \times 50 \times 10^{-6} \times 50}{y \cdot x} \times 100$$

where,

x Weight of the substance taken (g)

y Volume of the extract taken for P determination (ml)

z Reading from the standard curve (ppm) against percent transmission recorded

Volume of the extractant added = 50 ml

Volume made after colour developed = 50 ml

Preparation of standard curve

Prepare standard curve using 0.1 to 0.6 ppm *P* in 50 ml volumetric flask. Plot the standard curve by taking concentration of soluble *P* on x-axis and percent *T* on y-axis using a semi-log graph paper. It is a straight-line relationship between the soluble *P* and percent *T* when plotted on a semi-log graph paper.

Annex I
(normative)

Determination of the number of Azospirillum cells

Medium

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

Malic acid	5.0 g
Potassium hydroxide	4.0 g
Di-potassium hydrogen phosphate	0.5 g
Ferrous sulphate	0.05 g
Manganese sulphate	0.01 g
Magnesium sulphate	0.1 g
Sodium chloride	0.2 g
Calcium chloride	0.1 g
Sodium molybdate	0.002 g
Distilled water	1000 ml
Bromothymol blue (0.5 % alcoholic solution)	2.0 ml
Agar	1.7 g
Ph	Adjusted to 6.5 – 7.0

Preparation of serial dilution for MPN count

G.2.1 Dispense 30 g of Azospirillum inoculants in 270 ml of sterile water and shake for 10 minutes on a reciprocal shaker. Make serial dilutions up to 10-7 dilution.

G.2.2 Pipette out 0.2 ml aliquots of 10-4 to 10-8 dilution and deliver it to screw cap tubes or petridishes containing set medium as described in E - 2.1. Spread the aliquots over the plates. Invert the plates and place them in the incubator at 28 ± 2°C for 3 days. Use 5 replicates of 10-5, 10-6 and 10-7 level.

Counting

G.3.1 Count the tubes or plates which have turned blue in colour after inoculation and ascertain the presence of pellicles in undisturbed medium. To determine usual contamination on the same, examine doubtful objects carefully.

G.3.2 Count all plates/tubes which have turned blue and consider them for the purpose of calculation. Count such type of tubes/plates and tally this count with MPN table to get the number of cells per gram of the carrier.

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

Annex J
(normative)

Test for effective root growth

Pot Culture Test

H.1.1 For the purpose of 'Pot Culture Test' plant nutrition solution, preparation of solutions and procedure shall be same as described in C.2.1.1 and C.2.1.2 of Annex C.

H.1.2 After three weeks of growth, thin down the number of plants in each pot to four uniform plants. At the end of three months, take one set of pot from control and inoculated series and separate the plants carefully from the soil under slow running water. Obtain the number, length and mass of the plant as a whole including branch, seeds if any and roots.

H.1.3 At the end of three months, harvest the shoot system (depending on the crop). Dry at 60 °C for 48 hours and determine the dry mass. For the above purpose maintain a minimum of four replications or a maximum of 16 replications.

H.1.4 Better vigour of roots in inoculated seeds if established from the data when compared to the control, is the confirmation of effectiveness of ASI. If there is 10% increase in the plants over the dry mass of uninoculated control without nitrate, it may be concluded that the culture is of required quality.

Method for estimating MPN count for Azospirillum

H.2.1 Add 100 g ASI sample to 900 ml of sterile distilled water.

H.2.2 Shake for 10 minutes on a reciprocal shaker.

H.2.3 Make ten-fold dilution series.

H.2.4 Pipette 1 ml of each dilution (from 10^{-1} to 10^{-7}) to each one of 5 replicates (5 tubes containing nitrogen free bromothymol blue semi-solid malate media for Azospirillum).

H.2.5 Begin by taking aliquot from the highest dilution and proceed down the series with same pipette

H.2.6 Collect the tubes from 10^{-5}, 10^{-6} and 10^{-7} dilution level.

H.2.7 Incubate the inoculated series for 2 days at 30°C.

H.2.8 Check the characteristic sub-surface while particles (+ or -) in malate semi-solid medium, a change to dark blue colour of the medium.

Calculation

H.3.1 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 dilution.

H.3.2 Then find the row of numbers in Table E.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.

H.3.3 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.

Tables H.1 — Most Probable Numbers for use with 10 Fold dilutions and S Tubes per dilution (Cochran, 1950)

		Most probable number for indicated values of P_2					
P_1	P_2	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	0.40	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	-

Annex K
(normative)

Total plate count of Azotobacter

Preparation of culture Medium

E.1.1 Use a plating medium of the following composition

Agar	20.0 g
Sucrose ($C_{12}H_{22}O_{11}$)	20.0 g
Ferric sulphate Fe_2 (SO_4)$_3$	0.1 g
Dibasic potassium phosphate (K_2HPO_4)	1.0 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5 g
Sodium Chloride (NaCl)	0.5 g
Calcium carbonate ($CaCO_3$)	2.0 g
Sodium Molybdate (Na_2MoO_4)	0.005 g
Distilled water	1000 ml
Ph	6.8 to 7.2

E.1.2 Use nutrient agar (Beef extract 3.0g, tryptone 5.0 g, agar 15.0 g and distilled water 1000 ml, pH 7.0) for total count of bacteria. The extent of contamination can be assessed by difference between the total bacterial count and Azotobacter chroococum count.

E.1.3 The culture medium shall be prepared in accordance with ISO 11133 or using any other acceptable validated test method.

Preparation for serial dilution for plate count

E.2.1 Add 30 g of Inoculants to 270 ml of sterile distilled or demineralized water and shake for 10 min on a reciprocal shaker or homogenizer.

E.2.2 Make serial dilutions up to 10^{-9}. Take 0.1 ml or suitable aliquots of 10^{-6} to 10^{-9} dilutions using sterile pipettes and deliver to Petri dishes containing set medium as given in E.1.2 and spread it uniformly with a spreader. Invert the plates and promptly place them in the incubator.

Incubation of plates

Label the plates and incubate at 28±1 °C for 4 to 6 days.

Colony counting aids

Same as in C.4. (annex E)

Colony count

E.5.1 *Azotobacter chroococcum* colonies are gummy, raised with or without striations, viscous and often sticky. The pigmentation varies from very light brown to black. Count the colony number and observe the

cyst formation as given below and calculate number per gram of the carrier material.

E.5.2 Grow the vegetative cells at 30 °C on Burks agar medium comprising sucrose 20 g, dipotassium hydrogen phosphate 0.64 g, dihydrogen potassium phosphate 0.20 g, sodium chloride 0.20 g, calcium sulphate 0.05 g, sodium molybdate 0.001 g, ferric sulphate 0.003 g, agar 20 g and distilled water 1000 ml. Look for vegetative cells after 18 to 24 h either by simple staining method or through a phase contrast microscope.

E.5.3 Grow the cyst cells on Burks agar medium as given above with 0.3% n-butanol in place of the carbon source. Look for cyst formation after 4 to 5 days incubation.

Annex L
(normative)

Test for nitrogen fixation in pure culture

Preparation of pure culture medium

Prepare medium as given in E.1, excluding agar.

Procedure

Select from each Azotobacter colony, of the type that has been counted as *Azotobacter chroococcum*. Pick up one colony and plate on the medium given in (E.1). Use this pure culture for inoculating the broth for nitrogen fixation. For this purpose, take 50 ml aliquots of broth in 250 ml conical flasks for inoculation.

Inoculate and incubate for 7 days on a rotary shaker at 29 ± 1 °C. Test the contents of the flasks for purity by streaking on fresh medium. Concentrate the contents over water bath (50 to 60 °C) or in an oven at 90 °C or under vacuum at 60 °C to dryness.

Collect the dried culture and take it as a sample.

The contents of the flasks in inoculated control series (flasks inoculated and kept in deep freeze to inhibit growth) should be prepared in a similar manner to serve as control.

Determination by Kjeldahl method

Reagents

F.3.1.1 Sulphulic acid, 93 – 98 %, N-free

F.3.1.2 Mercuric oxide, N-free

F.3.1.3 Sodium hydroxide solution, N-free: Dissolve about 450 g of solid sodium hydroxide in water, cool and dilute to 1 L (specific gravity of the solution should be at least 1.36)

F.3.1.4 Potassium sulphate (or anhydrous sodium sulphate)

F.3.1.5 Sulphide or thiosulphate solution: Dissolve 40 g commercial potassium sulphides in 1 L of water (or solution of 40g of sodium sulphide or 80g of sodium thiosulphate in 1L may be used).

F.3.1.6 Zinc granules-reagent grade.

F.3.1.7 Indicators

F.3.1.8 Methyl red indicator: Dissolve 1g of methyl red in 200 ml of Ethanol.

F.3.1.9 Mixed indicator: Prepare mixed indicator by Dissolving 0.8 of methyl red and 0.2 g of methyl blue in 500 ml of ethanol.

F.3.1.10 Hydrochloric or sulphuric acid: Standard solution 0.5 N or 0.1 N when amount of nitrogen is small.

F.3.1.11 Boric acid, 4% solution

NOTE Ratio of salt to acid (m/v) is about 1:1 at the end of the digestion for proper temperature control. Digestion may be incomplete at a lower ratio, and nitrogen may be lost at higher ratio. Each gram of fat consumes 10 ml of sulphuric acid and each gram of carbohydrate 4.0 ml of sulphuric acid during digestion.

Apparatus

F.3.2.1 For digestion

F.3.2.1.1 Use kjeldahl's flasks of hard, moderately thick, well annealed glass with total capacity approximately 500 to 800 ml. Conduct digestion over heating device adjust to bring 250 ml of water at 25 °C to rolling boil in about 5 minutes. To test the heaters, preheat for 10 minutes in the case of gas burners and for 30 minutes in the case of electric heaters.

F.3.2.1.1 Add 3 to 4 boiling chips to prevent superheating.

F.3.2.2 For distillation

F.3.2.2.1 Use 500 to 800ml Kjeldahl's flask fitted with rubber stopper through which passes the lower end of an efficient scrubber bulb or trap to prevent mechanical carry-over of sodium hydroxide during distillation.

F.3.2.2.2 Connect the upper end of the bulb tube to a condenser by rubber tubing.

F.3.2.2.3 Trap the out let of the condenser in such a way as to ensure absorption of ammonia distilled over with the receiver.

Procedure

F.3.3.1 Place 0.25 g of the sample in the digestion flask. Add 0.7 gm mercuric oxide, 15 gm potassium sulphate followed by 25 ml of sulphuric acid. Shake, let stand for about 30 minutes and heat carefully until frothing ceases. Boil briskly until the solution clears and continue boiling further for 90 minutes.

F.3.3.2 Cool, add about 200 ml of water cool to room temperature and add a few zinc granules.

F.3.3.3 Tilt the flask and carefully add 50 ml of sodium hydroxide solution without agitation. Immediately connect the flask to the distillation bulb on the condenser whose tip is immersed in 50 ml of standard 0.1 N acid in the receiving flasks. Rotate the digestion flask carefully to mix the content. Heat until 150 ml of the distillate collects and titrate excess acid with 0.1 N base using methyl-red or mixed indicator. Carry out blank determination on reagents.

NOTE Check the ammonia recording periodically, using inorganic nitrogen control, for example, ammonium sulphate.

Calculation

$$\text{Nitrogen content, (\% by mass)} = \frac{\text{T.V.} \times \text{N} \times 1.4}{\text{m}}$$

Where;

T.V. titration value (ml of standard acid)

N normality of standard acid, and

M mass of sample taken.

Determination of sucrose consumed

Sample preparation

F.4.1.1 Take 1g of accurately weighed prepared sample of AI (F.2) into 250 ml volumetric flask and dilute with about 150 ml of water.

F.4.1.2 Mix thoroughly the contents of flask and make the volume to 250 ml with water. Centrifuge the mixture for 15 min at 10 000 rpm.

F.4.1.3 Decant the supernatant carefully and estimate the total reducing sugar after inversion (F.4.2)

Method of inversion

F.4.2.1 To 100 ml of the supernatant (F.4.1) add 1 ml of concentrated hydrochloric acid and heat the solution to near boiling. Keep aside overnight.

F.4.2.2 Neutralize this solution with sodium carbonate and determined the total reducing sugars.

Determination of reducing sugars

F.4.3.1 Reagents

F.4.3.1.1 Copper sulphate solution (Solution A): Dissolve 34.639 g of copper sulphate crystals (CuSO4.5H5O) in water, dilute to 500 ml and filter through glass wool or filter paper.

F.4.3.1.2 Potassium sodium tatrate (Rochelle salt) (Solution B): Dissolve 173g of potassium sodium tartrate and 50g of sodium hydroxide in water, and diltute to 500 ml. Let the solution stand for a day and filter.

F.4.3.1.3 Hydrochloric acid, specific gravity 1.18 at 20 °C (approximately 12N)

F.4.3.1.4 Standard invert sugar solution

F.4.3.1.4.1 Weigh accurately 0.95g of sucrose and dissolve it in 500 ml of water.

F.4.3.1.4.2 Add 32 ml of concentrated hydrochloric acid, boil gently for 30 min and keep aside for 24 h.

F.4.3.1.4.3 Neutralize with sodium carbonate and make the final volume to 1000 ml, 50 ml of this solution contains 0.05g of invert sugar.

F.4.3.1.5 Methylene blue indicator - 0.2% in water

F.4.3.1.6 Standardization of copper sulphate solution:

F.4.3.1.6.1 Using separate pipettes, pipette accurately 5 ml of solution A (**F.4.3.1 a**) and 5 ml of solution B (F.4.3.1 b) into a conical flask of 250 ml capacity.

F.4.3.1.6.2 Heat this mixture to boiling on asbestos gauze and add standard invert sugar solution (F.4.3.1 d) from a burette, about 1 ml less than the expected volume, which will reduce the Fehling solution completely (about 48 ml).

F.4.3.1.6.3 Add 1 ml of methylene blue indicator while keeping the solution boiling. Complete the titration within 3 min. The end point being indicated by change of colour from blue to red.

F.4.3.1.6.4 From the volume of invert sugar solution used, calculate the strength(**S)** of the copper sulphate solution by multiplying the titre value by 0.001 (g/ml of the standard invert sugar solution). This would give the quantity of invert sugar required to reduce the copper in 5 ml of copper sulphate solution.

F.4.3.2 Procedure

F.4.3.2.1 Using separate pipettes, pipette accurately 5 ml of solution A (F.4.3.1 a) and 5 ml of solution B (F.4.3.1 b) in porcelain dish.

F.4.3.2.2 Add abour 12 ml of Al solution from a burette and heat to boiling over a gauze.

F.4.3.2.3 Add 1 ml of methylene blue indicator and while keeping the solution boiling complete the titration within 3 minutes, the end point being by change of colour from blue to red.

F.4.3.2.4 Record the volume (**H**) in ml of Al solution required for titration.

F.4.3.2.5 Follow the same step steps for control.

Calculation

Total reducing sugars

$$\text{Total reducing sugars, (\% m/m)} = \frac{250 \times 100 \times S}{H \times M}$$

Where,

S strength of copper sulphate solution

H volume in ml of Al solution required for titration, and

M mass in g of Al taken for the test

Determination of sucrose

$$\text{Sucrose (\% m/m)} = [\text{(reducing sugars after inversion) (\%m/m) - (reducing sugars before inversion) (\%m/m)}] \times .95$$

Determination of nitrogen fixed per g of sucrose consumed

$$\text{Nitrogen fixed (mg)/g sucrose consumed} = \frac{\text{Nitrogen content (\%m/m) (F.3.4)}}{\text{Sucrose consumed (F.5.2)}}$$

Bibliography

- EAS 456, Organic production standard.
- US 1576, Bio fertilizers — Specification
- KS 2356, Bio fertilizers — Specification