GURU NANAK COLLEGE

DEPARTMENT OF PLANT BIOLOGY AND PLANT BIOTECHNOLOGY

SEMESTER V

Course title: PLANT BIOTECHNOLOGY

PRACTICAL MANUAL

Course code: 16UPBTC15P

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SEMESTER – V

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EXPERIMENT NO.: 1
Sterilization techniques in plant tissue culture

AIM: Aseptic culture techniques for establishment and maintenance of cultures

PRINCIPLE: Maintenance of aseptic environment: All culture vessels, media and instruments used in handling tissues as well as the explants must be sterilized. The importance is to keep the air surface and floor free of dust. All operations are carried out in laminar air-flow, a sterile cabinet.

Infection can be classified in three ways: 1. The air contains a large quantity of suspended microorganisms in the form of fungal and bacterial spores.

2. The plant tissue is covered with pathogens on its surface.

3. The human body (a skin, breathe etc) carries several microorganisms. In general, the methods of elimination of these sources of infection can be grouped under different categories of

Sterilization procedures:

1. Preparation of sterile media, culture vessels and instruments (sterilization is done in autoclave)
2. Preparation of sterile plant growth regulators stocks (by filter sterilization)
3. Aseptic working condition
4. Explants (isolated tissues) are sterilized using chemical sterilents, e.g. $HgCl_2$ and NaOCl.

Sterilization: It follows that all the articles used in the plant cell culture must be sterilized to kill the microorganisms that are present.

A. Steam or Wet sterilization (Autoclaving): This relies on the sterilization effect of superheated steam under pressure as in a domestic pressure cooker. The size of the equipment used can be as small as one litre or even as large as several thousand litres.

Most instruments/ nutrient media are sterilized with the use of an autoclave and the autoclave has a temperature range of 115- 1350 C. The standard conditions for autoclaving has a temperature of 1210 C and a pressure of 15 psi (Pounds per square inch) for 15 minutes to achieve sterility.

This figure is based on the conditions necessary to kill thermophilic microorganisms.

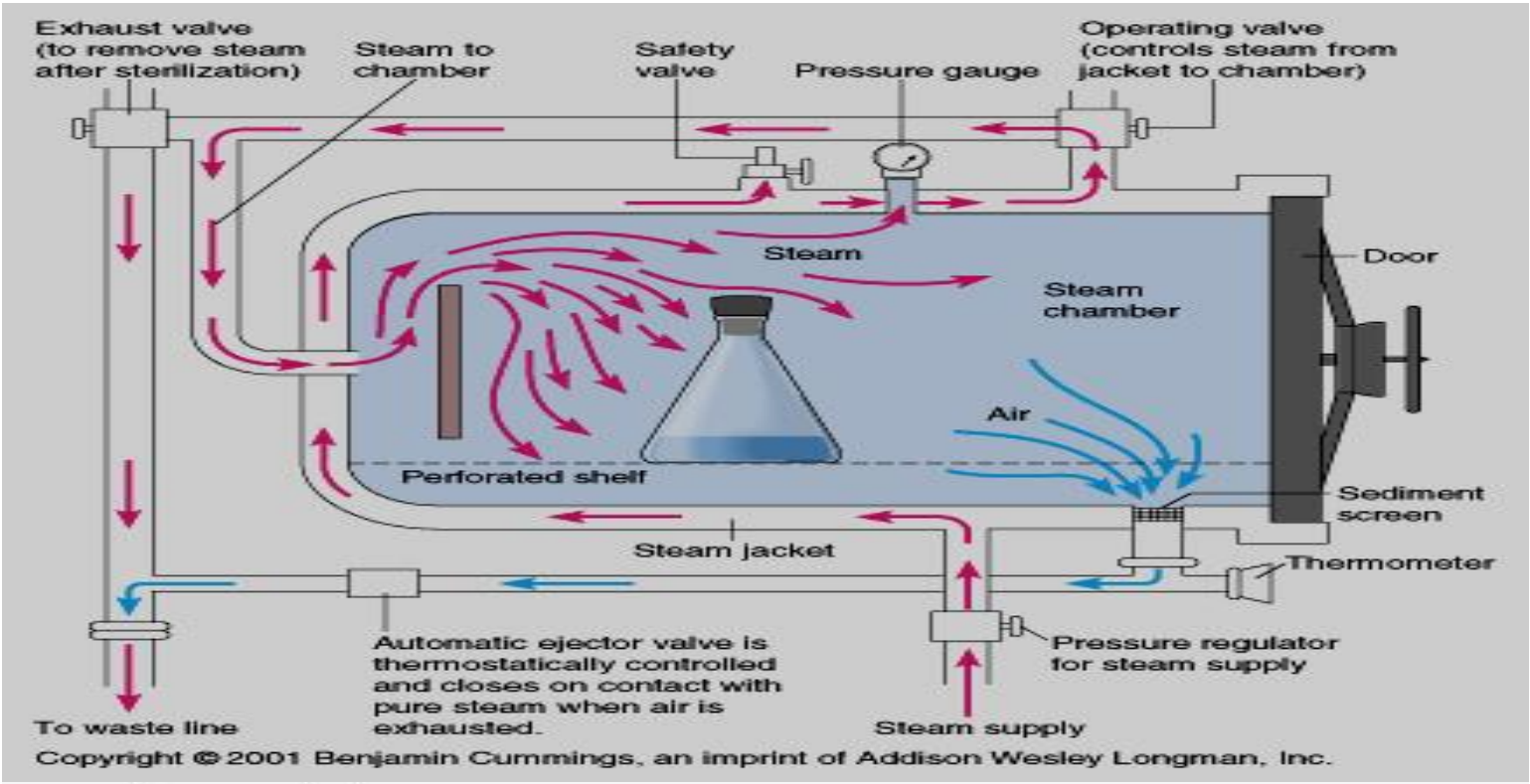
The time taken for liquids to reach this temperature depends on their volume.

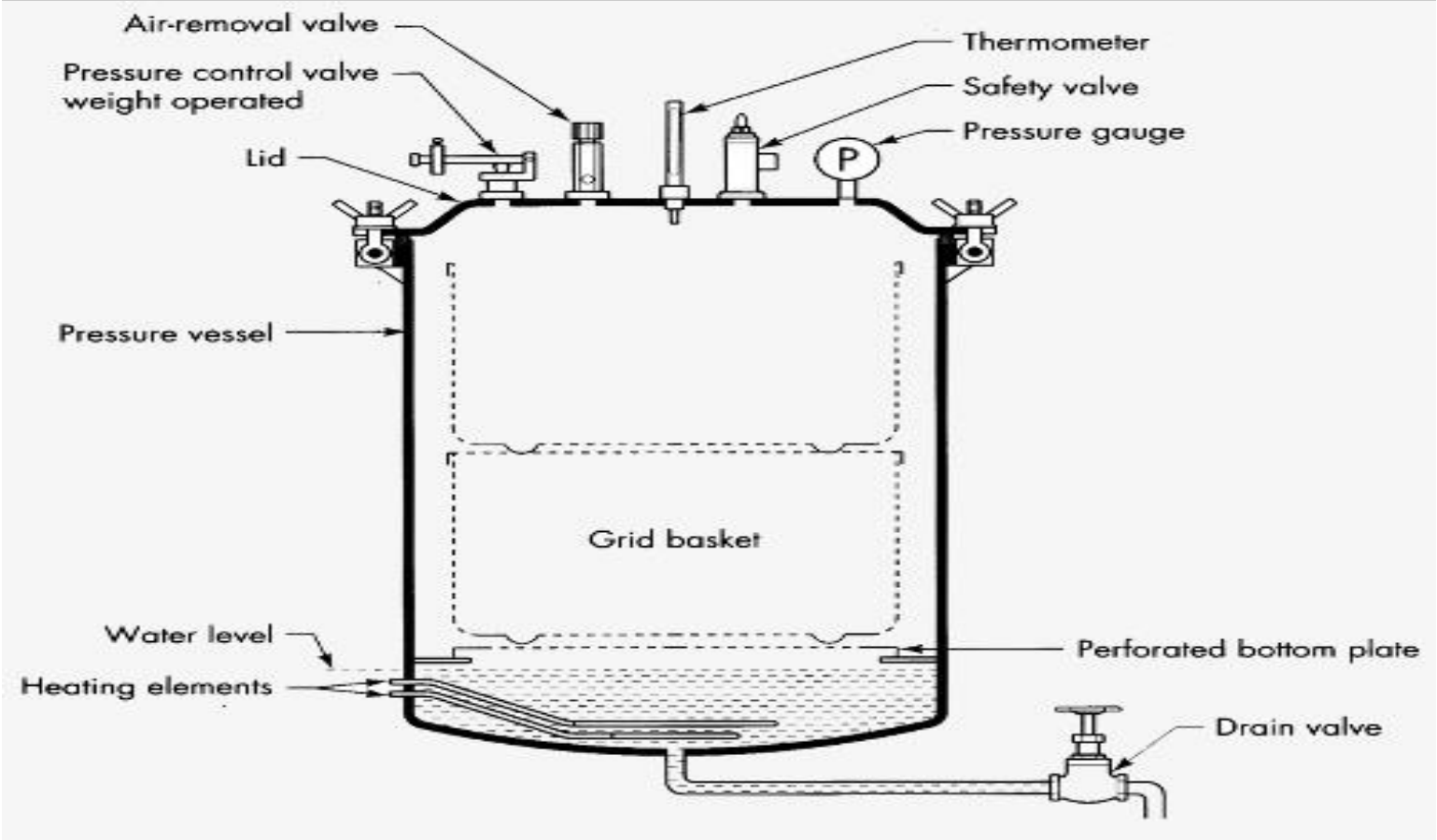
It may also depend on the thickness of the vessel.

The temperature of 1210 C can only be achieved at 15 psi.

The efficiency of autoclave can be checked in several ways: The most efficient way is to use an autoclave tape.

When the autoclave tape is autoclaved, a reaction causes dark diagonal strips to appear on the tape indicating that it is autoclaved.





Steam or Wet sterilization Autoclave

B. Filter sterilization:

Some growth regulators like amino acids and vitamins are heat labile and get destroyed on autoclaving with the rest of the nutrient medium.

Therefore, it is sterilized by filtration through a sieve or a filtration assembly using filter membranes of 0.22 µm to 0.45µm size.

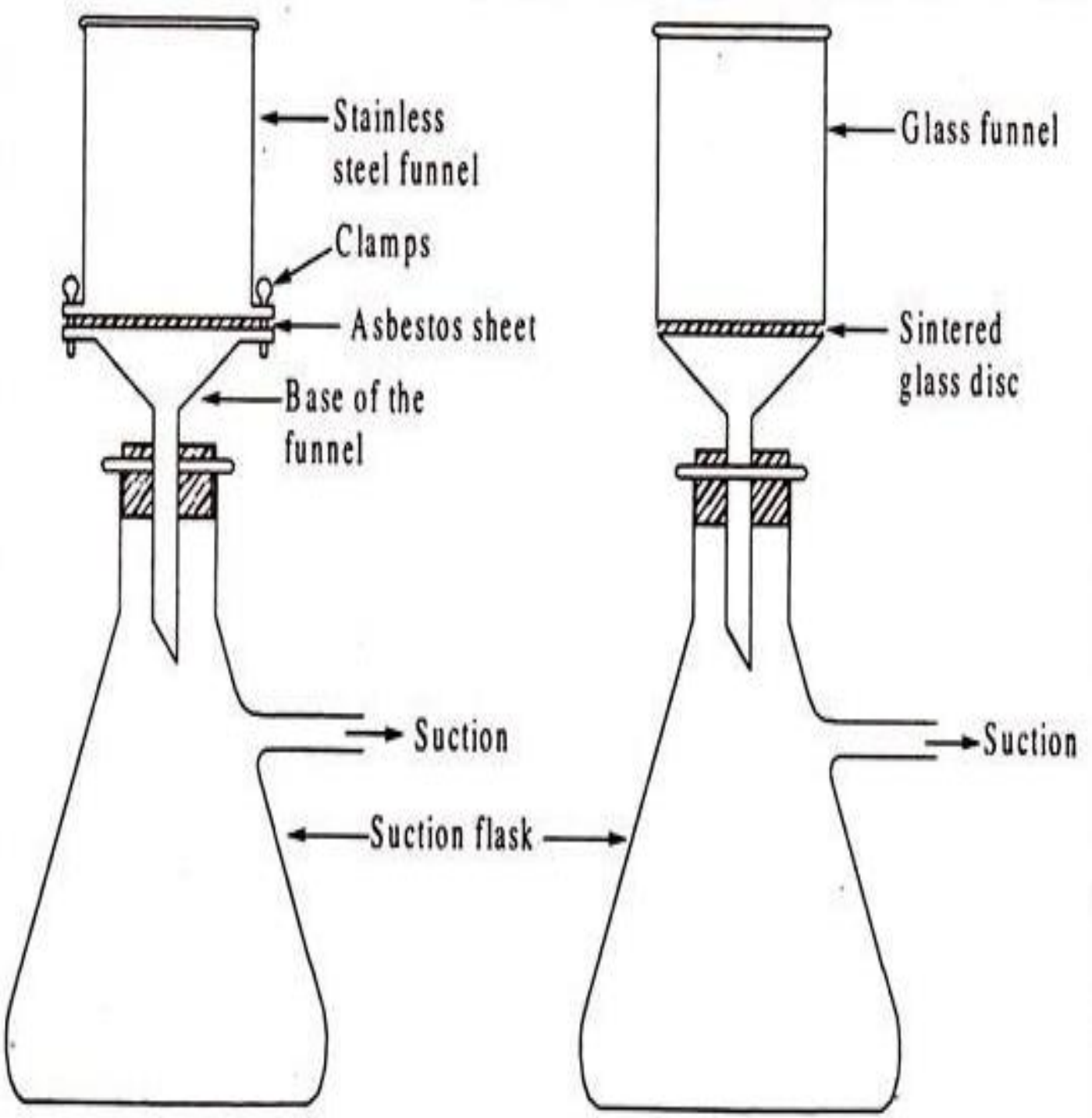
C. Irradiation: It can only be carried out under condition where UV radiation is available.

Consequently, its use is restricted generally to purchased consumables like petridishes and pipettes.

UV lights may be used to kill organisms in rooms or areas of work benches in which manipulation of cultures is carried out.

It is however, dangerous and should not be turned on while any other work is in progress.

UV light of some wavelengths can damage eyes and skin.



A. Seitz filter

B. Sintered glass filter

D. Laminar Airflow Cabinet: This is the primary equipment used for aseptic manipulation.

This cabinet should be used for horizontal air-flow from the back to the front, and equipped with gas corks in the presence of gas burners. Air is drawn in electric fans and passed through the coarse filter and then through the fine bacterial filter (HEPA).

HEPA or High Efficiency Particulate Air Filter is an apparatus designed such that the air-flow through the working place flows in direct lines (i.e. laminar flow). Care is taken not to disturb this flow too much by vigorous movements.

Before commencing any experiment it is desirable to clean the working surface with 70% alcohol.

The air filters should be cleaned and changed periodically.

Laminar Airflow Cabinet

Hot Air Oven : It is used to sterilize equipment and materials used in the medical field.

A hot air oven is a type of dry heat sterilization.

Dry heat sterilization is used on equipment that cannot be wet, and on material that will not melt, catch fire, or change form when exposed to high temperatures.

Moist heat sterilization uses water to boil items or steam them to sterilize and does not take as long as dry heat sterilization.

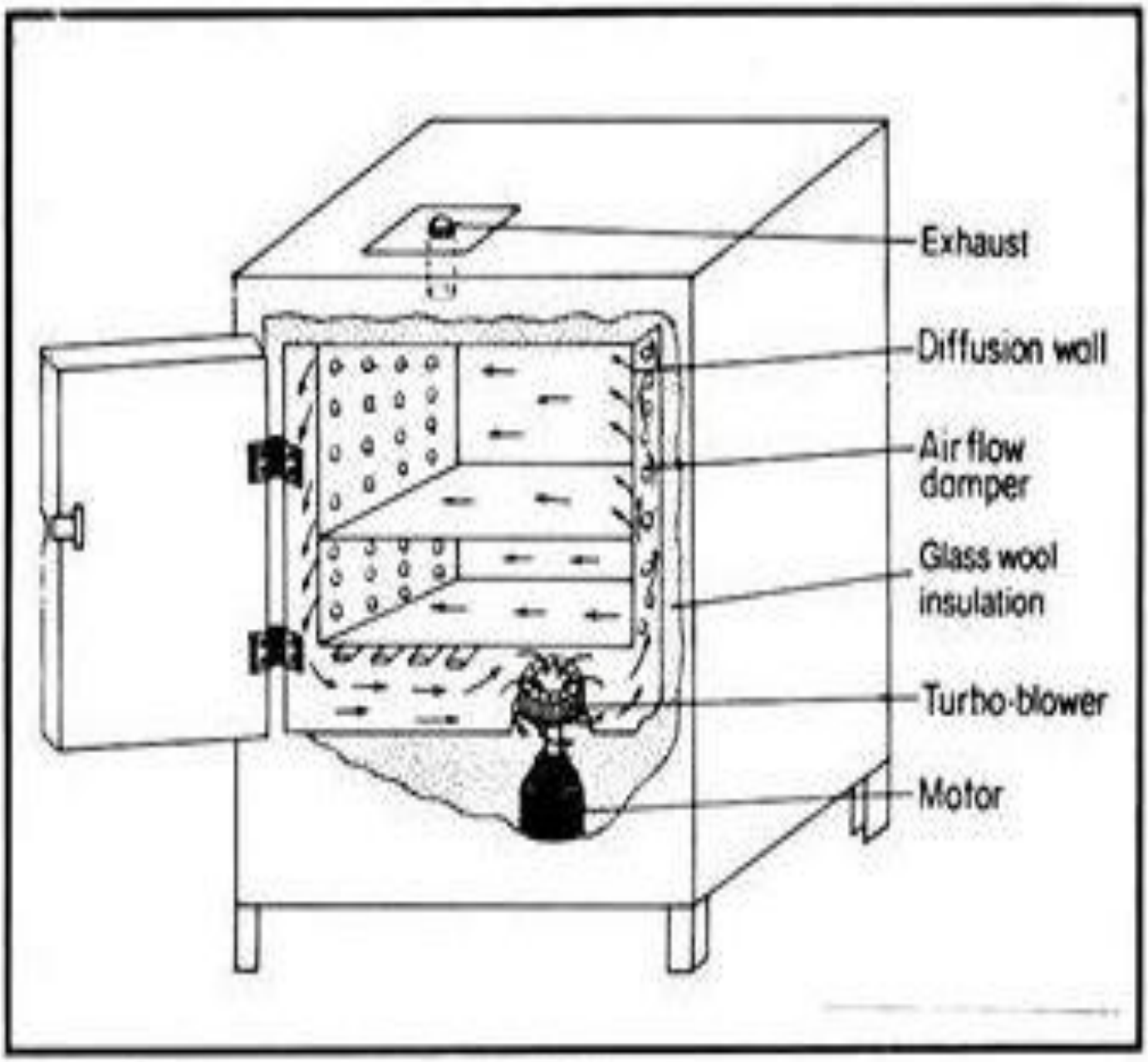
Examples of items that are not sterilized in a hot air oven are surgical dressings, rubber items, or plastic material.

Items that are sterilized in a hot air oven include:

- Glassware (petri dishes, flasks, pipettes, and test tubes)
- Powders (starch, zinc oxide, and sulfadiazine)
- Materials that contain oils
- Metal equipment (scalpels, scissors, and blades)
- Glass test tubes can be sterilized using a hot air oven

- Hot air ovens use extremely high temperatures over several hours to destroy microorganisms and bacterial spores.
- The ovens use conduction to sterilize items by heating the outside surfaces of the item, which then absorbs the heat and moves it towards the center of the item.

The commonly-used temperatures and time that hot air ovens need to sterilize materials is 170 degrees Celsius for 30 minutes, 160 degrees Celsius for 60 minutes, and 150 degrees Celsius for 150 minutes.


Exhaust
Diffusion wall
Air flow damper
Glass wool insulation
Turbo-blower
Motor

Hot Air Oven :

EXPERIMENT- 2

AIM: Preparation of stock solutions of MS (Murashige & Skoog, 1962) basal medium and plant growth regulator stocks.

PRINCIPLE: The basal medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue.

The tissue culture medium consists of 95% water, macro- and micronutrients, vitamins, aminoacids, sugars.

The nutrients in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reactions. The macronutrients are required in millimolar (mM) quantities while micronutrients are needed in much lower (micromolar, μM) concentrations.

Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions.

Sugar is essential for *in vitro* growth and development as most plant cultures are unable to photosynthesize effectively for a variety of reasons.

Murashige & Skoog (1962) medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration.

Plant growth regulators (PGRs) at a very low concentration (0.1 to 100 μM) regulate the initiation and development of shoots and roots on explants on semisolid or in liquid medium cultures.

The auxins and cytokinins are the two most important classes of PGRs used in tissue culture. The relative effects of auxin and cytokinin ratio determine the morphogenesis of cultured tissues.

MATERIALS:

- Amber bottles
- Plastic beakers (100 ml, 500 ml and 1000 ml)
- Measuring cylinders (500 ml)
- Glass beakers (50 ml)
- Disposable syringes (5 ml)
- Disposable syringe filter (0.22 μm)
- Autoclaved eppendorf tubes (2 ml)
- Eppendorf stand
- Benzyl-aminopurine
- Naphthalene acetic acid

INSTRUCTIONS:

MS NUTRIENTS STOCKS

Nutrient salts and vitamins are prepared as stock solutions (20X or 200X concentration of that required in the medium) as specified. The stocks are stored at 4^0 C. The desired amount of concentrated stocks is mixed to prepare 1 liter of medium.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497

Table 1.

MS major salts	mg/1 L medium	500 ml stock (20X)
1. NH_4NO_3	1650 mg	16.5 gm
2. KNO_3	1900 mg	19 gm
3. $Cacl_2.2H_2O$	440 mg	4.4 gm
4. $MgSO_4.7H_2O$	370 mg	3.7 gm
5. KH_2PO_4	170 mg	1.7 gm

MS minor salts	mg/1 L medium	500 ml stock (200X)
1. H_3BO_3	6.2 mg	620 mg
2. $MnSO_4.4H_2O$	22.3 mg	2230 mg
3. $ZnSO_4.4H_2O$	8.6 mg	860 mg
4. KI	0.83 mg	83 mg
5. $Na_2MoO_4.2H_2O$	0.25 mg	25 mg
6. $CoCl_2.6H_2O$	0.025 mg	2.5 mg
7. $CuSO_4.5H_2O$	0.025 mg	2.5 mg

MS Vitamins	mg/1 L medium	500 ml stock (200X)
1. Thiamine (HCl)	0.1 mg	10 mg
2. Niacine	0.5 mg	50 mg
3. Glycine	2.0 mg	200 mg
4. Pyrodoxine (HCl)	0.5 mg	50 mg

Iron, 500ml Stock (200X)

Dissolve 3.725gm of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) in 250ml dH_2O. Dissolve 2.785gm of $FeSO_4.7H_2O$ in 250 ml dH_2O Boil Na_2EDTA solution and add to it, $FeSO_4$ solution gently by stirring.

PLANT GROWTH REGULATOR STOCK

The heat-labile plant growth regulators are filtered through a bacteria-proof membrane (0.22 μm) filter and added to the autoclaved medium after it has cooled enough (less than 60^0 C). The stocks of plant growth regulators are prepared as mentioned below.

Table 2.

Plant Growth Regulator	Nature	Mol. Wt.	Stock (1 mM)	Soluble in
Benzyl aminopurine	Autoclavable	225.2	mg/ ml	1N NaOH
Naphtalene acetic acid	Heat labile	186.2	mg/ ml	Ethanol

The desired amount of plant growth regulators is dissolved as above and the volume is raised with double distilled water. The solutions are passed through disposable syringe filter (0.22 μm). The stocks are stored at -20^0 C.

Table 3. Characteristics of growth regulators

Name	Chemical formula	Molecular weight	Solubility
p-Chlorophenoxy acetic acid	$C_8H_7O_3Cl$	186.6	96% ethanol
2,4-Dichlorophenoxy acetic acid	$C_8H_6O_3Cl$	221.0	96% ethanol, heated lightly
Indole-3 acetic acid	$C_{10}H_9NO_2$	175.2	1N NaOH/96% ethanol
Indole-3 butyric acid	C12H13NO2	203.2	1N NaOH/96% ethanol
α-Naphthalene acetic acid	C12H10O2	186.2	1N NaOH/96% ethanol
β-Naphthoxy acetic acid	C12H10O3	202.3	1N NaOH
Adenine	$C_5H_5N_5.3H_2O$	189.1	H_2O
Adenine sulphate	$(C_5H_5N_5)_2.H_2SO_4.2H_2O$	404.4	H_2O
Benzyl adenine 6 benzyl amino purine	C12H11N5	225.2	1N NaOH
N-isopentenyladenine (2 iP)	C10H13N5	203.3	1N NaOH
Kinetic	$C_{10}H_9N_5O$	215.2	1N NaOH
Zeatin	C10H13N5O	219.2	1N NaOH/1N HCl, heated lightly
Gibberellic acid	C19H22O6	346.4	Ethanol
Abscisic acid	$C_{15}H_20O_4$	264.3	1N NaOH
Colchicine	C22H25NO6	399.4	H_2O

Table 4 Preparation of stock solutions of Murashige and Skoog (MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrients (10x) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 . 7H_2O$	370	3700	
KH_2PO_4	170	1700	
Macronutrient (10x) Stock solution II			
$CaCl_2$ $2H_2O$	440	4400	100 ml
Micronutrients (100x) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 . 4H_2O$	22.3	2230	
$ZnSO_4 . 7H_2O$	8.6	860	
Kl	0.83	83	
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_45H_2O$	0.025	2.5	
$CoCl_2 . 6H_2O$	0.025	2.5	
Iron source			
Fe EDTA Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	4 ml
Sucrose	30,000	Added fresh	
Agar	8000	Added fresh	
pH 5.8			

Nutrient medium chart for preparation of culture medium

Constituents	**Stock solution (conc.)**	**Quantity required for 1 L**	**Quantity required for volume of medium under preparation (e.g. 500ml)**	**Remarks**
Macro stock solution I	10x	100ml	50 ml	
Macro stock solution II ($CaCl_2$)	10x	100 ml	50 ml	
Micro stock solution III	100x	10 ml	5 ml	
Iron-EDTA Na salt	Added fresh	40 mg	20 mg	
Vitamins				
Nicotinic acid	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Thiamine HCl	50 mg/100 ml	0.1 mg/1 = 0.2ml	0.1 ml	
Pyridoxine HCl	50 mg/100 ml	0.5 mg/l = 1ml	0.5 ml	
Myo-inositol	Added fresh	100 mg	50 mg	
Others				
Glycine	50 mg/100 ml	2 mg/l = 4ml	2.0 ml	
Growth regulators				
Sucrose	Added fresh	30 g	15 g	
Agar	Added fresh	8 g	4 g	
pH				

EXPERIMENT NO.: 3

Embryo Culture

Aim:

To isolate embryos of *Cicer aertinum* and perform *in vitro* culture

Requirement:

1. Sterilants - alcohol, $HgCl_2$, sodium hypochlorite
2. Nutrition medium reagents - MS basic salts and vitamins
3. Growth regulators – usually not required for embryogenesis
4. Plant Material- Embryo of *Cicer auritinum*
5. Culture tubes containing media
6. Sterile Petri dishes
7. Scalpel, blades, forceps knives and steel-dissecting needles
8. Sterile distilled water

Procedure:

1. The seeds were washed by submerging them in water with a few drops of detergent in a beaker and shake them by hand.
2. The embryo was teased and collected without any damage
3. It was washed with distilled water and then treated with 70% alcohol for 30 seconds.
4. This was followed by rinsing completely with distilled water and then transferred to 20% sodium hypochlorite, where it was left for 0 minutes.
5. Then the embryo was thoroughly rinsed with distilled water for 3 times and dried using the autoclaved tissue paper and inoculated in the culture tubes containing the MS medium.
6. The culture tubes were incubated at 25^{o}C under 16 h photoperiod for 2 to 3 weeks.

Result:

The plant was developed from inoculated embryo.

Induction of plant let from the embryo

EXPERIMENT NO.: 4

Meristem Culture

Most of the horticultural and forest crops are infected by systemic disease caused by fungi, viruses, bacteria, Mycoplasma and nematode. While plant infected with bacteria and fungi may respond to treatments with bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus infected plants. It is possible to produce disease free plants through tissue culture. Apical meristems in the infected plants are generally either free or carry a very low concentration of the viruses. The various reasons attributed to the escape of the meristems by virus invasion are:

a) Viruses move readily in a plant body through the vascular system which in meristems is absent, b) A high metabolite activity in the actively dividing meristematic cells does not allow virus replication and

c) A high endogenous auxin level in shoot apices may inhibit virus multiplication. Meristem –tip cultures has also enabled plants to be freed from other pathogens including Viroids, mycoplasmas, bacteria and fungi. Therefore, main objective of shoot-tip and meristem –tip culture is the production of disease free plants through micro propagation.

Shoot-tip Culture:

It may be described as the culture of terminal (0.1-1.0mm) portion of a shoot comprising the meristem (0.05 -0.1) together with primordial and developing leaves and adjacent stem tissue.

Meristem Cultures:

Meristem cultures is the in vitro culture of a generally shiny special dome like structure measuring less than 0.1mm in length and only one or two pairs of youngest leaf primordia, most excised from the shoot apex.

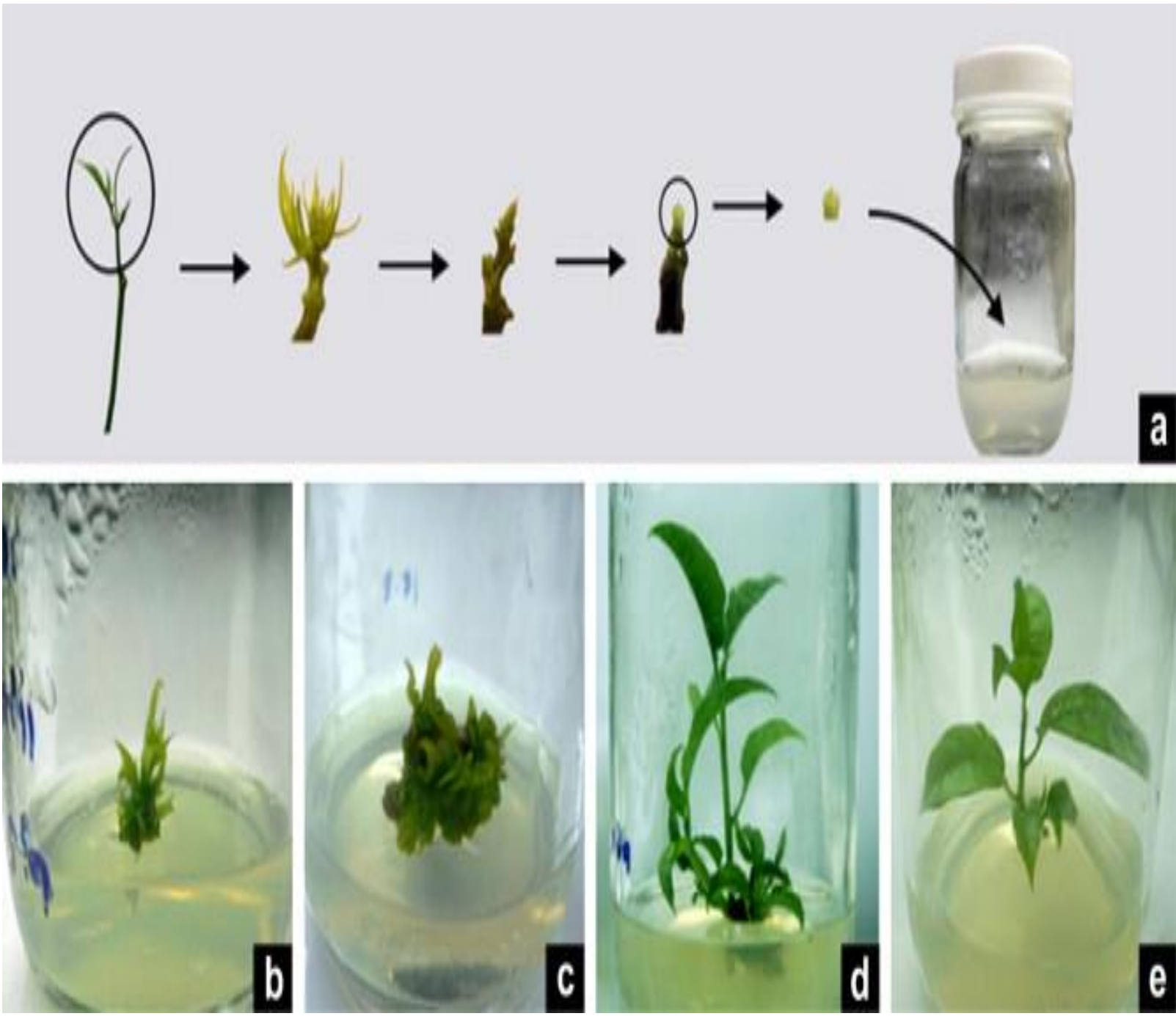
Principle:

The excised shoot tip and meristem can be cultured aseptically on agar solidified simple nutrient medium or on paper bridges dipping into liquid medium and under appropriate conditions will grow out directly into a small leafy shoot or multiple shoots. Alternatively, the meristem may form a small callus at its cut base on which a large number of shoot primordia will develop. These shoot primordia grow out into multiple shoots. Once the shoot have been grown directly from the excised shoot tip or meristem, they can be propagated further by nodal cuttings. This process involves separating the shoot into small segment each containing one mode. The axillary bud on each segment will grow out in culture to form a yet another shoot. The excised stem tips of orchids in culture proliferate to form callus from which some organised juvenile structures known as protocorm develop. When the protocorm are separated and cultured on fresh medium, they develop into normal plants. The stem tips of Cuscuta reflexa in culture can be induced to flower when they are maintained in the dark.

Exogenously supplied cytokinins in the nutrient medium plays a major role for the development of a leaf shoot or multiple shoots from the meristem or shoot tip. Generally high cytokinins and low auxin are used in combination for the culture of shoot tip of meristem. Addition of adenine suifate in the nutrient medium also induces shoot tip multiplication in some areas. BAP is the most effective cytokinins commonly used in shoot tip or meristem culture. Similarly, NAA is most effective auxins used in shoot tip culture. Coconut milk and gibberlic acid are also equally effective for the growth of shoot apices in some cases.

Protocol:

1. Remove the young twings from the healthy plant. Cut the tip portion of the twig.
2. Surface sterilize the shoot apices by incubation in a sodium hypochlorite solution (1% available chlorine) for 10 minutes. The explants are thoroughly rinsed 4 times in sterile distilled water.
3. Transfer each explant to a sterilize petridish.
4. Remove the outer leaves from each shoot apices with pair of jweller's forceps. This lessens the possibility of cutting into the softer underlying tissues.
5. After the removal of all the outer leaves, the apex is exposed. Cut off the ultimate apex with the help of scalpel and transfer only those less than 1 mm in length to the surface of the agar medium or to the surface of Filter Paper Bridge. Flame the neck of culture tube before and after the transfer of excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.
6. Incubate the culture under 16 hrs light at 25 0C.
7. As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, transfer them to hormone free medium to develop roots.
8. The plants form by this way are later transferred to pots containing compost and kept under green house condition for hardening.



Meristem Culture

EXPERIMENT NO.: 6

ANTHER CULTURE

AIM:

To isolate and inoculate anthers for haploid production.

PRINCIPLE:

Haploids refer to those plants which possess a gametophytic number of chromosomes in their sporophytes. Haploids may be grouped into two broad categories:

(a)monoploids which possess half the number of chromosomes from a diploid species.
(b)Polyhaploids which possess half the number of chromosomes from a polyploidy species.

Haploid production through anther culture has been referred to as androgenesis while gynogenesis is the production of haploid plants from ovary or ovule culture where the female gamete or gametophyte is triggered to sporophytic development.

MATERIALS REQUIRED:-

1. Anthers from *Hibiscus*
2. MS medium
3. growth factors
4. 70% ethanol
5. 2% mercuric chloride
6. Meso inositol
7. Scissors
8. Scalples
9. Petriplates
10. Forceps.

PROCEDURE:

1. Flower buds of *Hibiscus* were collected.

2. The flower buds are surface sterilized by immersing in 70% ethanol for 60 sec followed by immersing in 2% sodium hypochloride solution for 1 min or in mercuric chloride.

3. The buds were washed four or five times with sterile distilled water.

4. The buds were transferred to a sterile Petridish.

5. The buds were split open using a blade and the anthers were removed without damage and the filaments were removed.

6. The anthers were placed horizontally on the MS medium supplemented with different concentration of plant growth regulators or mesoinositol.

7. The Petriplates were sealed and incubated in dark at 28ºC.

8. The Petriplates were examined for the germination of anthers.

RESULT:

The anther underwent germination leading to the formation of haploid plantlets.

Anthers inoculated on the MS medium

EXPERIMENT NO.: 6
Study of algal biofertilizers

Biofertilizers

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants.

Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil.

Use of biofertilizers is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers. They can be grouped in different ways based on their nature and function.

S. No.	Groups	Examples
N2 fixing Biofertilizers		
1.	Free-living	*Azotobacter, Beijerinkia, Clostridium, Klebsiella, Anabaena, Nostoc,*
2.	Symbiotic	*Rhizobium, Frankia, Anabaena azollae*
3.	Associative Symbiotic	*Azospirillum*
P Solubilizing Biofertilizers		
1.	Bacteria	*Bacillus megaterium* var. *phosphaticum, Bacillus subtilis* *Bacillus circulans, Pseudomonas striata*
2.	Fungi	*Penicillium sp, Aspergillus awamori*
P Mobilizing Biofertilizers		
1.	Arbuscular mycorrhiza	*Glomus sp.,Gigaspora sp.,Acaulospora sp.*, *Scutellospora sp. & Sclerocystis sp.*
2.	Ectomycorrhiza	*Laccaria sp., Pisolithus sp.*, *Boletus sp.*, *Amanita sp.*
3.	Ericoid mycorrhizae	*Pezizella ericae*
4.	Orchid mycorrhiza	*Rhizoctonia solani*
Biofertilizers for Micro nutrients		
1.	Silicate and Zinc solubilizers	*Bacillus* sp.
Plant Growth Promoting Rhizobacteria		
1.	Pseudomonas	*Pseudomonas fluorescens*

Different types of biofertilizers

Rhizobium

Rhizobium is a soil habitat bacterium, which can able to colonize the legume roots and fixes the atmospheric nitrogen symbiotically. The morphology and physiology of Rhizobium will vary from free-living condition to the bacteroid of nodules. They are the most efficient biofertilizer as per the quantity of nitrogen fixed concerned. They have seven genera and highly specific to form nodule in legumes, referred as cross inoculation group.

Rhizobium inoculant was first made in USA and commercialized by private enterprise in 1930s and the strange situation at that time has been chronicled by Fred (1932).

Initially, due to absence of efficient bradyrhizobial strains in soil, soybean inoculation at that time resulted in bumper crops but incessant inoculation during the last four decades by US farmers has resulted in the build up of a plethora of inefficient strains in soil whose replacement by efficient strains of bradyrhizobia has become an insurmountable problem.

Azotobacter

A. chroococcum happens to be the dominant inhabitant in arable soils capable of fixing N2 (2-15 mg N2 fixed /g of carbon source) in culture media.

The bacterium produces abundant slime which helps in soil aggregation. The numbers of *A. chroococcum* in Indian soils rarely exceeds 105/g soil due to lack of organic matter and the presence of antagonistic microorganisms in soil.

Azolla

- *Azolla* is a free-floating water fern that floats in water and fixes atmospheric nitrogen in association with nitrogen fixing blue green alga *Anabaena azollae*.
- *Azolla* fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots.
- Rice growing areas in South East Asia and other third World countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers.
- *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N/ha per rice crop.


Phosphatesolubilizing microorganisms(PSM)

Several soil bacteria and fungi, notably species of *Pseudomonas, Bacillus, Penicillium, Aspergillusetc*. secrete organic acids and lower the pH in their vicinity to bring about dissolution of bound phosphates in soil. Increased yields of wheat and potato were demonstrated due to inoculation of peat based cultures of *Bacillus polymyxa* and *Pseudomonas striata*. Currently, phosphate solubilizers are manufactured by agricultural universities and some private enterprises and sold to farmers through governmental agencies. These appear to be no check on either the quality of the inoculants marketed in India or the establishment of the desired organisms in the rhizosphere.

Azospirllium

It belongs to bacteria and is known to fix the considerable quantity of nitrogen in the range of 20- 40 kg N/ha in the rhizosphere in non- non-leguminous plants such as cereals, millets, Oilseeds, cotton etc. The efficiency of *Azospirillium* as a Bio-Fertilizer has increased because of its ability of inducing abundant roots in several pants like rice, millets and oilseeds even in upland conditions. Considerable quantity of nitrogen fertilizer up to 25-30 % can be saved by the use of *Azospirillum* inoculant. The genus *Azospirillum* has three species viz., *A. lipoferum*, *A. brasilense* and *A. amazonense*. These species have been commercially exploited for the use as nitrogen supplying Bio-Fertilizers.

One of the characteristics of *Azospirillum* is its ability to reduce nitrate and denitrify. Both *A. lipoferum*,and *A. brasilense* may comprise of strains which can actively or weakly denitrify or reduce nitrate to nitrite and therefore, for inoculation preparation, it is necessary to select strains which do not possess these characteristics. *Azospirllium lipoferum* present in the roots of some of tropical forage grasses uch as Digitaria, Panicum, Brachiaria, Maize, Sorghum, Wheat and Rye.

Physical features of liquid *Azospirillum*

•The colour of the liquid may be blue or dull white.

•Bad odours confirms improper liquid formulation and may be concluded as mere broth.

•Production of yellow gummy colour materials comfirms the quality product.

•Acidic pH always confirms that there is no Azospirillum bacteria in the liquid.

N2 fixing capacity of Azospirillum in the roots of several plants and the amount of N2 fixed by them.

Plant	Mg N2 fixed /g of substrate
Oryza sativa (Paddy)	28
Sorghum bicolour (Sorghum)	20
Zea mays (Maize)	20
Panicum sp.	24
Cynodon dactylon	36
Setaria sp	12
Amaranthus spinosa	16

Production of growth hormones

Azospirillum cultures synthesize considerable amount of biologically active substances like vitamins, nicotinic acid, indole acetic acids giberllins. All these hormones/chemicals helps the plants in better germination, early emergence, better root development.

Role of Liquid *Azospirillum* under field conditions

Stimulates growth and imparts green colour which is a characteristic of a healthy plant.

Aids utilization of potash, phosphorous and other nutrients.

Encourage plumpness and succulence of fruits and increase protein percentage.

Sign of non functioning of *Azospirillum* in the field

No growth promotion activity

Yellowish green colour of leaves, which indicates no fixation of Nitrogen

VAM Fungi:

- Production of VAM inoculum has evolved from the original use of infested field soils to the current practice of using pot culture inoculum derived from the surface disinfected spores of single AM fungus on a host plant grown in sterilized culture medium.
- Several researches in different parts of the world resulted in different methods of production of AM fungal inoculum as soil based culture as well as carrier based inoculum.
- Root organ culture and nutrient film technique provide scope for the production of soil less culture.
- As a carrier based inoculum, pot culture is widely adopted method for production.
- The AM inoculum was prepared by using sterilized soil and wide array of host crops were used as host.
- The sterilization process is a cumbersome one and scientists started using inert materials for production of AM fungi.

Nursery application: 100 g bulk inoculum is sufficient for one metre square. The inoculum should be applied at 2-3 cm below the soil at the time of sowing. The seeds/cutting should be sown/planted above the VAM inoculum to cause infection.

For polythene bag raised crops: 5 to 10 g bulk inoculum is sufficient for each packet. Mix 10 kg of inoculum with 1000 kg of sand potting mixture and pack the potting mixture in polythene bag before sowing.

For out –planting: Twenty grams of VAM inoculum is required per seedling. Apply inoculum at the time of planting.

For existing trees: Two hundred gram of VAM inoculum is required for inoculating one tree. Apply inoculum near the root surface

Method of VAM production

1. Tank for mass multiplication of AM

2. Sprinkling of water in tank with vermiculite

3. Making of furrows to sow maize seeds

Mass production of VAM inside Bricklined tank

Mass production of VAM inside a cement tank

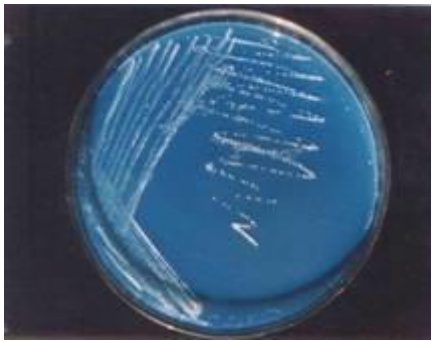
Azospirillum

Azospirillum lipoferum and *A. brasilense* (*Spirillum lipoferum* in earlier literature) are primary inhabitants of soil, the rhizosphere and intercellular spaces of root cortex of graminaceous plants. They perform the associative symbiotic relation with the graminaceous plants.

The bacteria of Genus *Azospirillum* are N2 fixing organisms isolated from the root and above ground parts of a variety of crop plants. They are Gram negative, *Vibrio* or *Spirillum* having abundant accumulation of polybetahydroxybutyrate (70 %) in cytoplasm.

Five species of *Azospirillum* have been described to date *A. brasilense*, *A.lipoferum*, *A.amazonense*, *A.halopraeferens* and *A.irakense*. The organism proliferates under both anaerobic and aerobic conditions but it is preferentially micro-aerophilic in the presence or absence of combined nitrogen in the medium.

Apart from nitrogen fixation, growth promoting substance production (IAA), disease resistance and drought tolerance are some of the additional benefits due to *Azospirillum* inoculation.



Azospirllium

It belongs to bacteria and is known to fix the considerable quantity of nitrogen in the range of 20-40 kg N/ha in the rhizosphere in non- non-leguminous plants such as cereals, millets, Oilseeds, cotton etc.

The efficiency of *Azospirillium* as a Bio-Fertilizer has increased because of its ability of inducing abundant roots in several pants like rice, millets and oilseeds even in upland conditions. Considerable quantity of nitrogen fertilizer up to 25-30 % can be saved by the use of *Azospirillum* inoculant.

The genus *Azospirillum* has three species viz., *A. lipoferum*, *A. brasilense* and *A. amazonense*.

These species have been commercially exploited for the use as nitrogen supplying Bio-Fertilizers.

One of the characteristics of *Azospirillum* is its ability to reduce nitrate and denitrify.

Both *A. lipoferum*,and *A. brasilense* may comprise of strains which can actively or weakly denitrify or reduce nitrate to nitrite and therefore, for inoculation preparation, it is necessary to select strains which do not possess these characteristics.

Azospirllium lipoferum present in the roots of some of tropical forage grasses uch as Digitaria, Panicum, Brachiaria, Maize, Sorghum, Wheat and Rye.

Physical features of liquid *Azospirillum*

•The colour of the liquid may be blue or dull white.

•Bad odours confirms improper liquid formulation and may be concluded as mere broth.

•Production of yellow gummy colour materials comfirms the quality product.

•Acidic pH always confirms that there is no Azospirillum bacteria in the liquid.

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