ALAGAPPA UNIVERSITY

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KARAIKUDI – 630 003

DIRECTORATE OF DISTANCE EDUCATION

M.Sc. BOTANY

III SEMESTER

34634

Practical: - Microbiology & Plant Pathology, Ecology, Biodiversity Conservation, Economic Botany, Algal Technology and Mushroom Technology

34634PRACTICAL –LAB III:MICROBIOLOGY & PLANT PATHOLOGY, ECOLOGY, BIODIVERSITY CONSERVATION, ECONOMIC BOTANY, ALGAL TECHNOLOGY AND MUSHROOM TECHNOLOGY

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1. Microbiology & Plant Pathology

1.1 GRAM STAINING OF BACTERIA

Aim: To perform gram staining of the given sample.

Material Required: Glass slides, Bunsen burner, cotton, sample, and microscope.

Reagents Required: Crystal violet dye, iodine, alcohol (95% ethyl alcohol), saffranin dye

Principle: Gram staining is most widely staining technique used in m/o examination. It was discovered by Danish scientist and physician Hans Christain Joachin Gram in 1884. This technique differentiates bacteria in 2 groups i.e. Gram positive and Gram negative bacteria. The procedure is based on the ability of m/o to retain colour of the stain during Gram reaction. Gram negative bacteria are decolourised by alcohol losing the colour of primary stain, purple. Gram positive bacteria are not decolourised by alcohol and will remain as purple. After decolourisation stop , a counter stain is used to impart pink colour to the gram negative m/o. Gram positive bacteria have a thick mesh like cell wall which is made up of peptidoglycan (50-90%) of cell wall, which stain purple. Gram negative bacteria have a thinner layer of peptidoglycan (10% of cell wall) and lose the crystal violet iodine complex during decolourisation with alcohol rinse but retain the counter stain saffranin thus appearing reddish or purple.

Stain reaction:

1. Application of crystal violet to heat fixed smear: CV dissociates in aqueous solution into CV+ and Cl- ions. These two penetrate the cell wall and cell membrane of both gram positive and gram negative. CV+ interact with negative component of bacterial cell and stain it purple.

2. Addition of gram iodine: Iodine acts as a mordant and a trapping agent. A mordant is substances that increase the affinity of cell wall for a stain by binding to primary stain, thus forming a insoluble complex that get trapped in cell. During the reaction CV-I compex is formed and all the cells turn purple.

3. Decolourization with ethyl alcohol: Alcohol dissolve the lipid outer membrane of gram negative bacteria, thus leaving the petidoglycan layer exposed and increase the porosity of cell wall. The CV-I complex is then washed away from the peptidoglycan layer leaving gram negative bacteria colourless.In gram positive bacteria , alcohol has dehydrating effect on cell wall causing cell wall to shrink , then CV-I complex get tightly bound into multi layered leaving the cell with purple colour.

4. Counter stain with saffranin dye: The decolourised gram negative cell can be visible with a suitable counter stain which is usually positively charged saffranin, which stained it pink.

Procedure:

1. Prepared very thin smear of sample on glass slide and heat fixed it.
2. Flooded the smeared slide with crystal violet dye. Avoid over flooding and kept it for 1 minute.
3. Washed the slide under running tap water.
4. Applied iodine solution gently all over the slide and kept for 1 minute.
5. Washed it under tap water.
6. Applied 95% ethyl alcohol all over the slide drop wise and kept for 10 second.
7. Immediately rinsed with water.
8. Finally, flooded the sample with saffranin dye to counter stain and kept for 45 seconds.
9. Washed the slide with running water.
10. Observed it under microscope.

1.2 STERILIZATION METHODS, PREPARATION OF MEDIA AND STAINS.

Aim: To know different methods of Sterilization and their laboratory practices

Principle:

Sterilization can be achieved by a combination of heat, chemicals, irradiation, high pressure and filtration like steam under pressure, dry heat, ultraviolet radiation, gas vapor sterilants, chlorine dioxide gas etc. Effective sterilization techniques are essential for working in a lab and negligence of this could lead to severe consequences, it could even cost a life.

Procedure:

Heat Method: This is the most common method of sterilization. The heat is used to kill the microbes in the substance. The extent of sterilization is affected by the temperature of the heat and duration of heating. On the basis of type of heat used, heat methods are categorized into-

(i) Wet Heat/Steam Sterilization- In most labs, this is a widely used method which is done in autoclaves. Autoclaves use steam heated to 121–134 °C under pressure. This is a very effective method that kills/deactivates all microbes, bacterial spores and viruses. Autoclaving kills microbes by hydrolysis and coagulation of cellular proteins, which is efficiently achieved by intense heat in the presence of water. The intense heat comes from the steam. Pressurized steam has a high latent heat and at 100°C it holds 7 times more heat than water at the same temperature. In general, Autoclaves can be compared with a typical pressure cooker used for cooking except in the trait that almost all the air is removed from the autoclave before the heating process starts. Wet heat sterilization techniques also include boiling and pasteurization.

(ii) Dry heat sterilization- In this method, specimens containing bacteria are exposed to high temperatures either by flaming, incineration or a hot air oven. Flaming is used for metallic devices like needles, scalpels, scissors, etc. Incineration is used especially for inoculating loops used in microbe cultures. The metallic end of the loop is heated to red hot on the flame. The hot air oven is suitable for dry material like powders, some metal devices, glassware, etc.

Filtration is the quickest way to sterilize solutions without heating. This method involves filtering with a pore size that is too small for microbes to pass through. Generally filters with a pore diameter of 0.2 um are used for the removal of bacteria. Membrane filters are more commonly used filters over sintered or seitz or candle filters. It may be noted that viruses and phage are much smaller than bacteria, so the filtration method is not applicable if these are the prime concern.

Radiation sterilization: This method involves exposing the packed materials to radiation (UV, X-rays, gamma rays) for sterilization. The main difference between different radiation types is their penetration and hence their effectiveness. UV rays have low penetration and thus are less effective, but it is relatively safe and can be used for small area sterilization. X-rays and gamma rays have far more penetrating power and thus are more effective for sterilization on a large scale. It is, however, more dangerous and thus needs special attention. UV irradiation is routinely used to sterilize the interiors of biological safety cabinets between uses. X-rays are used for sterilizing large packages and pallet loads of medical devices. Gamma radiation is commonly used for sterilization of disposable medical equipment, such as syringes, needles, cannulas and IV sets, and food.

Chemical method of sterilization: Heating provides a reliable way to get rid of all microbes, but it is not always appropriate as it can damage the material to be sterilized. In that case, chemical methods for sterilization is used this involves the use of harmful liquids and toxic gases without affecting the material. Sterilization is effective using gases because they penetrate quickly into the material like steam. There are a few risks, and the chances of explosion and cost factors are to be considered.

The commonly used gases for sterilization are a combination of ethylene oxide and carbon-dioxide. Here Carbon dioxide is added to minimize the chances of an explosion. Ozone gas is another option which oxidize most organic matter. Hydrogen peroxide, Nitrogen dioxide, Glutaraldehyde and formaldehyde solutions, Phthalaldehyde, and per acetic acid are other examples of chemicals used for sterilization. Ethanol and IPA are good at killing microbial cells, but they have no effect on spores.

Preparation of culture media

Aim: To prepare culture media.

Requirements: Nutrient broth, Nutrient Agar, Distilled Water, Autoclave, flask etc.

Principle: Culture medium or the growth medium is liquid or gel designed to support the growth of microbes. Most common media used for culturing the micro-organism is nutrient broth. When mixed with agar and poured in petri plates, it solidifies and provide solid medium for microbial cultures. It remains solid as very few micro-organisms are able to decompose agar. It contains all the nutrients required by micro-organisms and is non selective.

Nutrient broth consists of:

Composition	**g/l**
Peptones	5
NaCl	5
Yeast Extract	2
Beef Extract	1

13g of nutrient broth for 10 ml of media is added. It is dissolved in distilled water to prepare 1000ml of media only if it is available otherwise nutrient broth can be supplemented with 2% of agar- agar to prepare nutrient agar.

Procedure:

1- Weighed point 0.6g of nutrient broth and mixed with 50ml of distilled water.

2- Cotton plug the flask

3- 4.2g of nutrient agar was weighed and it was added to 150ml of distilled water. Again cotton plugs the flask.

4- Nutrient broth and agar was autoclaved at 1210C for 15minutes at 15 psi pressure.

5- After autoclaving, the media was cooled to 450C.

Preparation of Simple stains

Aim: To prepare and study the bacterial cell by simple techniques of staining

Principle:

The simple stain can be used as a quick and easy way to determine cell shape, size and arrangements of bacteria. True to its name, the simple stain is a very simple staining procedure involving single

solution of stain. Any basic dye such as methylene blue, saffranin, or crystal violet can be used to color the bacterial cells.

These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since the surface of most bacterial cells and cytoplasm is negatively charged, these positively charged stains adhere readily to the cell surface. After staining, bacterial cell morphology (shape and arrangements) can be appreciated.

Procedure:

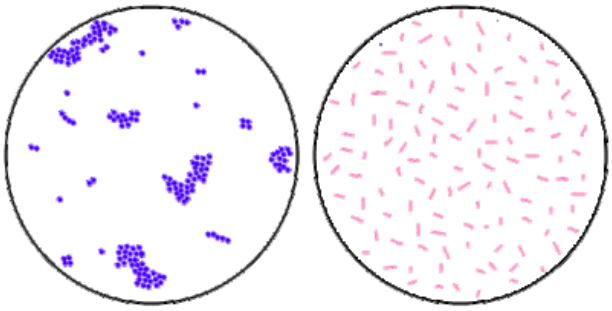
Preparation of a smear and heat fixing

1. Using a sterilized inoculating loop, transfer loopful of liquid suspension containing bacteria to a slide (clean grease free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
2. Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.
3. Allow the smear to dry thoroughly.
4. Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.

Staining

1. Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).
2. Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
3. Wash off any stain that got on the bottom of the slide as well.
4. Saturate the smear again but this time with Iodine. Iodine will set the stain

5. Wash of any excess iodine with gently running tap water. Rinse thoroughly. *(You may not get mention about step 4 and 5 in some text books)*
6. Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.
7. Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.
8. Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply immersion oil directly to the smear, and focus the smear under oil with the 100X objective.



Left: Cocci in Cluster; Right: Bacilli (Image source: *microrao.com*)

1.3. SYMPTOMOLOGY OF SOME DISEASED SPECIMENS

AIM: To study the symptoms of various diseases caused by infectious microbes in different types of plant specimens.

White rust

White rust or white blisters disease is one of the common diseases of crucifer crops. It is worldwide in distribution occurring in all the areas wherever crop is cultivated. Both wild and cultivated varieties are attacked.

The disease affects a large number of crucifer crops of economic importance like Mustard, Cress, Rape, Radish, Cabbage, Cauliflower, turnip etc. In India the disease is reported on Mustard, Rape, *Eruca sativa*, turnip. Cauliflower and *Cleome viscosa*.

Causal Organism:

The causal organism *Albugo candida* (Lev.) Kunze or *Cystopus candidus* Lev. is an obligate parasite.

Symptoms of White Rust Disease:

The disease affects all the aerial parts of the plant, the roots are not attacked. Symptoms may appear as a result of two types of infection: Local and Systemic.

In case of local infection, isolated spots or pustules appear on leaves or stems or inflorescence. The pustules are of variable size, measuring 1 -2 mm in diameter and are raised shiny white areas.

These may arise in close proximity and coalesce to form large irregular patches. Usually, the pustules appear in circular or concentric arrangement with one or two central areas. The host epidermis ruptures exposing white powdery mass consisting of spores of the fungus. Pustules occurring on leaves are usually confined to the lower surface only.

In systemic infections, young stems and inflorescence are infected. The fungus becomes systemic in these parts and the affected tissues are stimulated to various types of deformities. The most prominent is Hypertrophy of the affected parts. Due to Hypertrophy and Hyperplasia of floral parts, these show swellings and distortion.

The peduncle and pedicel may become enormously thickened upto 12-15 times, the normal diameter. Floral parts become fleshy, swollen, green or violet in colour, the stamens falling off early.

The petal may turn green sepal like and stamens and carpels are also converted to swollen leaf like structures. The ovules are usually atrophied as also the pollen grains resulting in total sterility. Pustules may also appear on these parts. However, the affected parts are full of oospores and starch.

When the systemic infection has taken early, the growth of the entire plant is checked, stunted and only small leaves may be formed.

Downy Mildew

Description of Downy Mildew of Grape:

This is one of the best known of the many diseases of plants of economic importance. It has an interesting historical background associated with the accidental discovery of Bordeaux mixture as a fungicide by the French plant pathologist, Millardet who perfected the Bordeaux mixture as a spray for this disease.

The best information available indicates that the downy mildew of grape is endemic in North America, where it was for the first time reported by Schweinitz in 1837. This disease was introduced in France in 1874 and subsequently in other parts of Europe by 1917, where it became a serious disease because of frequent epiphytotics. It is also well established in North and South Africa, Australia and New Zealand.

The disease is confined largely to species and varieties of grapes (Vitis) although it has been reported also on the five-leaved ivy (*Parthertocissus quinquefolia* Planch.), and on English ivy (*P. tricuspidata* Planch.).

Causal Organism of Downy Mildew of Grape:

Plasmopara viticola (Berk, and Curt.) Berl. The characteristic aseptate, intercellular mycelium produces knob-like haustoria to absorb food from the host cells. Fasciculate- sporangiophores arise from the hyphae in the intercellular spaces just beneath the lower epidermis and emerge through the stomata.

Symptoms of Downy Mildew of Grape:

The disease attacks all green parts of the plant leaf blades, petioles, tendrils, green shoots, and fruits at different stages of development. Early symptoms of the disease on the leaves consist of round light-green spots of an oily appearance on the upper surface, which enlarge even 1 /2 cm or more in diameter. On the corresponding under side white downy mildew consisting of the tufts of sporangiophores soon appear, bearing sporangia in great numbers.

In moist weather it persists; in extremely dry weather it may disappear. Later the spots become yellow, or variegated with tints of yellow and yellowish-brown forming patches of irregular shape,

especially between the larger veins of the leaf. Old spots turn brown owing to the killing of the leaf tissue.

The leaf spots may be few in number or so numerous as to, coalesce and involve nearly the entire leaf area. At this time the leaves are in a morbid condition, and the fungus within enters upon the sexual phase, producing oogonia, antheridia, and finally oospores which survive the winter in the fallen leaves.

On the stems, the lesions are brown and sunken, and along with the death of the affected parts, portions of the wine become brittle and break off easily. In extreme cases of infection the whole shoot may be dwarfed, the leaves remaining very small and densely covered with the mildew.

Flowers may be completely blighted by early attacks of the disease. Fruits may be attacked when young or when approaching maturity. The young fruits show brownish spots and later become covered with downy mildew, their growth is checked. The young fruits then darken and finally dry up.

They may also assume a reddish- brown colour and failing to ripen, develop a soft root. On full-grown fruits, brownish patches appear and the fruits harden ultimately becoming mummified. The disease may often strip plants of their leaves and tendrils, flowers may fail to set, fruit may be destroyed in the early stages of growth, causing severe loss.

Powdery Smuts

Introduction to the Corn Smut:

Corn smut disease is worldwide in its distribution. In India the disease occurs wherever com is grown particularly in the Punjab, U.P. and Kashmir. It is known as Kangiari.

Causal Organism:

The disease causing organism is *Ustilago maydis* (Dc) Cda. The black, generally spherical to ellipsoidal, heavily echinulate smut spores are the resting spores. They over-winter on the crop refuse in the soil or in the manure of cattle fed on the diseased corn.

Symptoms of Corn Smut Disease:

The disease is easily recognizable by the presence of large knob-like sooty swellings or boils known as the smut galls or balls tumors. No other symptoms appear on the host plant before the formation of smut ball. The latter appear on ears or stems (stalks).

Tumors are also formed on the leaves and tassels (male flowers). They are, however, much smaller in size. The galls at an early stage are light colored and are edible. At this stage they are covered with a firm shining membrane.

It is greenish white in colour. Towards maturity the tumours turn sooty due to spore formation inside. The covering membrane gradually dries and finally bursts to expose the sooty, powdery mass of smut spores.

The disease is localized and not systemic. Each tumour is the result of a separate and distinct infection. Unlike other cereal smuts the fungus mycelium does not pervade the entire plant. Infection takes place through the meristematic (embryonic) tissue of the over ground parts of the host plant such as stems, leaves, ears and tassels.

Corn smut thus provides an example of general infection through any embryonic tissue. Infection takes place any time during the growing season. In case the infection takes place through the silk, tumours are formed in place of kernels.

Ergot

Description of ergot:

Ergot is a fungal disease caused by fungi of the genus *Claviceps*. Species in this genus are unique in that they only infect ovaries of the host plants; no other part of the plant is infected. There are approximately 40 species of *Claviceps* with *C. purpurea* (Fries ex Fries) Tulasne being the species of greatest concern. Although *C. purpurea* has a very broad host range, including approximately 400 grass species, the most economically important of these is rye. Although ergot of rye causes yield reductions, the significance of the disease is primarily related to the toxic alkaloids present in the ergots (sclerotia). The alkaloids can cause

severe health problems in both humans and animals. Severe poisoning outbreaks as a result of ergot fungi are called ergotism.

Causal organism: *Claviceps purpurea*

Symptoms:

The first obvious sign of ergot infection is appearance of 'honeydew', a sticky yellow sugary solution consisting of host sap and conidia between the affected glumes of the rye. This is secreted by the infected plant ovary, which eventually is replaced by a purplish-black sclerotium, commonly referred to as an ergot. The size of the sclerotium depends on the host plant; it is generally 1 to 5 times larger than the host seed. Thus, the largest ergots (1-5 cm, 0.4-2 inches) are found in large-seeded plants such as cereal rye. The sclerotium consists of a whitish mycelial tissue containing storage cells and a dark pigmented outer cortex that protects the fungal mycelia from desiccation, UV light and other adverse environmental conditions.

Groundnut Leaf Spot

Introduction to the Tikka Diseases of Groundnut:

One of the best known leaf spot diseases is that of *Arachis hypogea* L. (groundnut). Popularly it is called the tikka disease. The tikka disease is a serious disease occurring in areas where the groundnut crop is grown in India.

The spots appear on the host leaves when the plants are one or two months old. Later necrotic lesions appear on the stem as well. In fact there are two leaf spot diseases of groundnut caused by two different species of form genus *Cercospora* namely, *C. arachidicola*.

The leaf spot disease caused by the former is more common, dangerous and does greater damage than the latter. The spot produced by *C. personata* are numerous. These weaken the host plant and lead to defoliation which adversely affects size and quality of the fruit.

Causal Organism:

Tikka disease of groundnuts is caused by two species of *Cercospora*: *Cercospora personata* (Berk. & Curt.) Elle and Eve, now known as *Cercosporidium personatum* (Berk. & Curt.) Deighton and

Cercospora arachidicola Hori. The two form-species differ from each other with respect to the size, shape and colour of necrotic lesions they produce, conidia formation and the nature of the mycelium.

Symptoms of Tikka Disease of Groundnut:

All parts of the host plant above soil level are attacked by the disease. The first visible symptoms appear on the leaflets of lower leaves as dark spots which at a later stage, are surrounded by yellow rings. The spots are circular. They appear in a large number on the leaves. Mature spots are dark-brown to almost black, particularly on the upper surface of the leaflets.

Whereas, on the lower surface they are lighter in colour. The spots are few on the leaf petioles and stem. Sometimes spots coalesce resulting in the defoliation. The shedding of leaves is a characteristic feature of the disease. Due to excessive spotting and consequent leaf fall, smaller and fewer nuts are formed.

In cases where young plants are attacked by the disease, nuts fail to develop in them. But the mature plants when attacked by the disease produce immature nuts which are shrivelled and become loose in the shell. The total effect is the loss in yield.

Red Rot of Sugarcane

Introduction to Red Rot of Sugarcane:

This is one of the most severe of the known diseases of sugarcane. It was first described from Java by Went in 1893. It is widely distributed throughout the sugarcane-growing countries of the world, and in fact it is extremely doubtful if there are any sugarcane-growing areas where it does not exist, although it may be much more destructive in some places than others.

The disease was very widespread and virulent in North Behar and Eastern part of the United Provinces during 1939 and 1942. It was so destructive that it almost whipped out the sugarcane plantations in those areas.

Causal Organism of Red Rot of Sugarcane:

Red rot of sugarcane disease is caused by *Colletotrichum falcatum* Went, the perfect stage of which is *Glomerella tucumanensis* (Speg.) Arx and Muller. Some insisted that this fungus is more strictly saprophytic than parasitic, and that it cannot attack healthy canes.

Symptoms of Red Rot of Sugarcane:

The first external evidences of disease are the drooping, withering, and finally yellowing of the upper leaves. This is followed by a similar wilting of the entire crown, and finally the entire plant shows indications of disease and dies. When not severe, the eyes frequently die and blacken and the dead areas extend out from the nodes.

Infection in the stem being internal, the presence of the disease is not visible externally. Upon splitting a diseased cane during the early stages of the disease, it will be found that the fibro-vascular bundles near the base are reddish in colour. The host tissue reacts vigorously to the presence of the fungus and some kind of reaction or change sets in the host cells in advance of the hyphal invasion.

The protoplasm changes colour and a gummy dark-red material oozes out of the cells filling the intercellular spaces. The soluble pigment present in this ooze, is absorbed by the cell wall producing the characteristic red rot appearance.

However, the presence of a red colour in the fibro-vascular bundles is not necessarily an indication of this disease, since the colour may be due to any one of many other causes. As the disease advances the red colour spreads to the surrounding tissues extending through many internodes and irregular discoloured blotches are formed, which may be reddish or yellowish or white with red margins.

These white areas with red margins are a positive proof of the disease. When the stem is completely rotted inside, the natural bright colour of the rind disappears and turns dull as it shrivels. Black specks appear on shrivelled rind. The stem shrinks at the nodes. Split cane gives sour smell and shows red tissue with white cross-bands.

About this time the upper leaves of the stem turn pale and gradually droop down. These leaves then wither at the tips and along the margins. Ultimately the entire plant withers and droops down. In areas where the disease appears in a severe epidemic form, the entire crop withers and droops resulting in a complete loss of crop.

Though the fungus attacks all parts of the host above ground, stems and midribs of leaves are more susceptible to fungal attack. Infection in the leaves is visible along the midribs as dark-reddish zones having tendency to elongate rapidly turning blood-red enclosed by dark margins. When the infection becomes old, the central blood-red colour changes to straw colour.

The hyphae after ramifying in the infected host tissue collect beneath the epidermis and form a stroma of densely packed cells and ultimately an acervulus is developed resulting in the rupture of host epidermis. The acervulus bears long septate setae along with short conidiophores on which falcate (sickle-shaped) conidia are borne.

After growing for a period within the host tissue, the hyphae produce a large number of chlamydospores in the pith parenchyma. The chlamydospores persist in the soil for a long time.

An examination of the diseased tissues with a microscope will reveal more or less mycelial threads of the fungus, or if the diseased canes are split and put in a moist chamber the fungus will develop readily and be easily recognized.

Wilt

Introduction to the Wilt of Arhar:

The wilt disease of arhar is a most severe threat to the arhar producing states in the country such as U.P., M.P., Bihar and in parts of Bombay.

Causal Organism:

The causal organism is *Fusarium oxysporum* Butler. The mycelium is septate and branched. It is colourless. The hyphae are both intercellular and intracellular.

Symptoms of Wilt Disease:

The susceptible plants are attacked when young about five to six weeks old. The symptoms are variable. Typically the first symptom is the premature yellowing of the leaves. The next symptom is the wilting or withering of the leaves of the diseased plants. The plant is in a blighted condition.

The leaves appear to be affected progressively from the bottom towards the top. Sudden wilting is rare. Finally the entire plant completely dries up. In severe cases of the infection more than half the plants in the field may fall victim to this disease.

The wilting is brought about by the plugging of the vascular tissue of the stem and roots of the host plant by dense masses of mycelial hyphae. The free flow of water to the leaves is thus interfered with.

This results in drooping and wilting of the leaves of the host plants. In addition the fungal hyphae produce toxic substances which kill the plant cells concerned in the ascent of sap. In cases where plugging of the vascular tissue of the host takes place on one side only, the wilting is partial.

Paddy Blast

Introduction to Rice Blast:

Rice (*Oryza sativa* L.) is the major staple food for nearly one half of the world's population. It occupies an area of 156.7 million hectare, with a total production of 650.2 million tons in 2007. India has an area of over 44.0 million hectare under rice, producing 144.1 million tons of paddy in 2007.

Rice cultivation is the principal activity and source of income for about 100 million household in Asia and Africa (FAO, 2004). It is primarily a tropical and subtropical crop, but the best grain yields are obtained in temperate regions.

The rice crop suffer from a number of diseases among them rice blast caused by one of the most devastating agricultural pathogens in the world, a fungus called *Magnapor grisea* (Hebert) Barr [anomorph: *Pyricularia grisea* (Cooke) Sacc.], is one of the most important, causing

significant losses in yield. Rice blast was probably first recorded as rice fever disease in China in 1637.

Symptoms of Rice Blast:

Rice blast is caused by *Magnaporthe grisea* (Hebert) Barr. All the above ground parts of the plant can be attacked by the fungus at any growth stages. However, Seedling stage, rapid tillering stage after transplanting and flower emergence stage were identified as the most susceptible ones to blast.

The disease can be described based on the part of the plant infected as follow:

i. Leaf Blast:

On the leaves the lesion/ spots first appear as minute brown specks, and then grow to become spindle-shaped, pointed at both ends. The center of the spots is usually gray or whitish with brown or reddish-brown margin. Fully developed lesions reach 1-1.5 cm long, 0.3-0.5 broad. Under favorable conditions, lesions enlarge and coalesce; eventually kill the leaves.

ii. Collar Rot:

Infection at the junction of the leaf blade and sheath in the typical brown "collar rot" symptom. A severe collar rot can cause the leaf to die completely. When collar rot kill the flag or penultimate leaf it may have a significant impact on yield.

iii. Neck Blast:

This occur when the pathogen infect the neck of the panicle to cause a typical **"neck rot"** or rotten neck blast symptom. The infected neck is griddled by a grayish brown lesion and the panicle falls down if the infection is severe. If the neck blast occurs before the milk stage, the entire panicle may die prematurely, leaving it white and completely unfilled.

iv. Panicle Blast:

The pathogen also causes brown lesions on the branches on the panicles and on the spikelets pedicles, resulting in **"panicle blast".** Infection of the neck, panicle branches, and spikelets pedicles may occur together or may occur separately.

v. Node Blast:

The fungus may also attack the stem at nodes, node blast in which the stem bend and break at the node causing spikelets sterility. Blast Symptoms of seeds themselves consist of brown spots, blotches.

Bacterial Blight of Paddy

Introduction to Bacterial Leaf Blight Disease of Rice:

Rice is the most important and staple food crop for more than two thirds of the population of India. The slogan "Rice is Life" is most appropriate for India as this crop plays a vital role in our national food security and is means of livelihood for millions of rural households. India has the largest acreage under rice (44.6 m.ha) and with a production of about 90 million tones it ranks second to China.

Causal Organism:

A number of modern approaches to bacterial taxonomy, classification and nomenclature seem to be promising especially with the bacterial blight pathogen. In 1908, Takaishi found bacterial masses in dew drops of rice leaves but he did not name the organism. Bokura in 1991 isolated a bacterium, and after a study of its morphology and physiology, the bacterium was named *Bacillus oryzae* Hori and Bokura.

Ishiyama (1922) studied the disease further and renamed the bacterium *Pseudomonas oryzae Uyeda* and *Ishiyama* according to Migula's system. It was later transferred to *Xanthomonas oryzae*.

According to the revision of the international code of nomenclature of bacteria the committee of taxonomy of phytopathogenic bacteria of the International Society of Plant Pathology adopted the name *Xanthomonas campestris* pv. oryzae Dye. In 1990, the pathogen was elevated to a species status and was named *Xanthomonas oryzae* pv. oryzae.

Symptomatology of Bacterial Leaf Blight Disease of Rice:

Bacterial blight has three significant symptoms viz. leaf blight, pale yellow and kresek. The leaf blight phase symptoms develop mostly on leaf blades, leaf sheaths and sometimes on grains. The symptom development depends upon the rice variety, physiological condition of rice plant, virulence of the pathogen and climatic conditions.

In rice seedlings small water soaked spots appear on the edges of lower leaves. These spots enlarge and gradually turn yellow. The leaves of the disease affected plants during tillering phase roll up, droop, turn yellow, or grayish brown and finally wither.

Leaf blight phase usually appears as tiny water soaked lesions at the fully developed leaves from the tip. The lesions enlarge both in length and width with a wavy margin and turn yellow within a few days. As the disease advances, in lesions cover the entire blade, turn white and later become grayish from the growth of various saprophytic fungi.

Lesion may also start at any point on the blade if it is injured. On the surface of young lesions, milky or opaque dry drops may be observed in the early morning. They dry up to form small, yellowish, spherical beads, which are easily shaken off by wind and drop into the field water.

The symptom of pale yellowing of rice leaves was first noted in the Philippines. Pale yellow leaves can be found on 3 week old seedlings when artificially inoculated in the field on plants that are tillering. While the older leaves are normal and green, the youngest leaf has either a yellow stripe on the blade or uniformly pale yellow.

On resistant cultivars, a yellow stripe appears just inside the margins of leaf blade, with no formation of necrotic lesions for some time. The stripes may eventually turn yellow and necrotic. On susceptible cultivars, infected blades wilt and roll as the diseased portion enlarges while the leaves are still green.

The entire blade may dry up. The lesion may also extend to the leaf sheath where they may reach to the lower end on these cultivars. In severe infection, symptom on the glumes appears as discolored spots surrounded by a water soaked halo.

Kresek (wilt) phase of the disease appears due to systemic infection of host by the pathogen. Initially it was described as a separate disease by Reitsma and Schure (1950) but later it was confirmed as an additional symptom of the bacterial blight. The term 'Kresek' is Indonesian means a resulting sound of withered leaves.

Initial symptom appears as green, water soaked spot just beneath the cut surface, which soon turn grayish green. Rolling and withering of the entire leaf including the leaf sheath occur later. The bacterium spreads through the xylem vessels and infects the base of other leaves. This is direct relationship of root injury and the kresek phase of the disease.

The greatest incidence of Kresek was reported to occur in 14 and 21 days old seedlings that were exposed to *X. oryzae* in the seedbed, 24 hours before transplanting. Goto (1964) identified the causal organism as *X. oryzae* and confirmed that kresek is one of the symptoms of the bacterial blight syndrome. The kresek phase of bacterial blight has been reported in the Philippines, Malaysia, India, Sri Lanka, China and Korea.

Reddy (1983) proved the movement of bacterium upward in rice seeding growing from infected seed, showing that the bacterial wilt is transmitted by seed. In the infected crown region of plant soft rot also develops which is extended to apical part of the clump in the older plants.

Angular Leaf Spot Cotton
Introduction:

Bacterial blight of cotton (also called angular leaf spot, boll rot, and black leg) is a potentially destructive bacterial disease for cotton production. Bacterial Blight, also called Angular Leaf Spot, is a disease caused by the bacterium, *Xanthomonas citri* pv. *malvacearum* ("Xcm" will be used throughout this bulletin). Bacterial Blight was first described in the United States in 1891 and continues to be a major disease of cotton throughout the world. In the U.S., commercially-planted cotton seed undergoes a process called "acid delinting," whereby the fibers that remain on the seed after ginning are removed by sulfuric acid. Before acid-delinted seed was the commercial standard, losses to Bacterial

Blight in some severely affected fields were as high as 60%. Since acid delinting has been implemented, losses to Bacterial Blight have been estimated at approximately 0.1% annually. However, losses can be much greater in individual fields, with recent reports of 20% in isolated instances. Historically, losses observed in Texas and Oklahoma has been greatest; but in recent years, Bacterial Blight has become increasingly problematic throughout the humid region of the Cotton Belt. Twenty-two races of Xcm have been described. A pathogen's race is defined by its ability to trigger susceptibility or resistance in different cotton varieties (and sometimes other plant species). Race 18 is the predominant race affecting cotton in the U.S. Should races other than 18 become more common, the effectiveness of the current, commercially-available resistant varieties may be negatively impacted.

Causal Organism:

Xanthomonas axonopodis pv. *malvacearum* (Smith) Vauterin.

Symptoms:

Small water-soaked spots appear on the under surface of cotyledons, which may dry and wither. Such spots also appear on the leaves. They become angular bound by veinlets and turn brown to black in colour. Several small spots may coalesce. The infected petiole may collapse. Elongated, sunken and dark brown to black lesions appear on stem, petioles and branches.

The young stems may be girdled and killed in the black arm phase. Sunken black lesions may be seen on the bolls. Young boll may fall-off. The attacked stem becomes weak. Bacterial slime is exuded on the brown lesions. Discolouration of lint may take place.

Tobacco Mosaic Virus

Introduction to Tobacco Mosaic Virus:

This is the best known of all virus diseases. The tobacco mosaic virus affects all dicotyledonous plants of which most important are tobacco and tomato. But it does not affect any monocotyledonous plants.

Although Adolph Mayer in 1886 first pointed out the mosaic pattern on leaves of affected tobacco plants, it was not until 1898 the first

scientific proof of the existence of a virus was given by Beijerinck. Earlier than this, in 1892 Iwanowski demonstrated that tobacco mosaic virus would pass through a bacteria-proof filter. He was able to demonstrate that a diseased tobacco plant juice was able to induce mosaic disease in healthy tobacco plants.

The tobacco mosaic virus affects photosynthetic tissue of the host leading to distortion, blistering and necrosis. It also causes dwarfing of affected plants. It is one of the most damaging viruses of plants, causes enormous loss of tobacco crop by reducing yield and quality.

Causal Organism of Tobacco Mosaic Virus:

The typical tobacco mosaic virus is Tobacco mosaic virus 1, *Marmor tabaci* Holmes. The virus remains active in extracted host plant juice even up to 25 years. It is a very resistant virus, can stand desiccation for 25 years or more. It occurs in very high concentration in plant and its dilution end point is 10^{-6}. The thermal inactivation point of the virus is 90°C.

Symptoms of Tobacco Mosaic Virus:

The symptom is systemic mosaic type. The primary symptom on young leaves is faint circular chlorotic lesions appear with gradual vein clearing. This is followed by the development of characteristic systemic mosaic. With the maturity of the leaves, abnormally dark-green spots appear which develop into irregular crumpled blister-like areas while the rest of the tissue becoming more or less chlorotic. Various degrees of leaf malformation like enations follow and some leaves exhibit only a mild diffuse mottle.

The development of symptoms is governed by many variable factors of which the most important is the difference in virulence of the virus strains.

For example, one strain of tobacco mosaic virus may cause yellow mottling on the leaves, a second may cause necrosis only, whilst a third induces a gross malformation. Another variable factor is the variety of plant affected. In flowers, petals show mosaic symptoms. Severe strains cause streaking of stem. The disease is seldom fatal to the host.

Little Leaf of Brinjal

Introduction:

Little leaf of brinjal is known to cause heavy economic losses in India. As the name indicates, symptoms of the disease include shortening of the petioles and production of leaves which are much smaller in size. Petioles are so short that leaves seem to be glued to the stem. They become soft and glabrous and somewhat yellow in colour. Affected plants do not bear any flowers or fruits if infection is in early stages of plant growth. However, in cases of late season infections fruits may remain small, become hard and unfit for consumption or marketing. The disease is caused by a plant pathogenic mollicute, Phytoplasma (earlier known as mycoplasma like organism or MLO) and is transmitted by the insect vector, *Hishimonus phycitis* which belongs to the group of leafhoppers. Management of the insect vector by means of insecticides and cultivation of resistant varieties are the principal means of management of the disease.

Causal Organism: *Hishimonus phycitis*

Symptoms of Little Leaf Disease:

The main symptom of the disease is the production of very short leaves by affected plant. The petioles are so much reduced in size that leaves appear sticking to the stem. Such leaves are narrow, soft, smooth and yellowish in colour.

Newly formed leaves are further reduced in size. The internodes are shortened and at the same time large number of axillary buds are stimulated to grow into short branches with small leaves. This gives whole plant a bushy appearance. Usually such plant unable to form flowers. Fruiting is very rare.

Sesame Phyllody

Introduction:

Phyllody is the abnormal development of floral parts into leafy structures. It is generally caused by phytoplasma or virus infections, though it may also be because of environmental factors that result in an imbalance in plant hormones. Phyllody causes the affected plant to

become partially or entirely sterile, as it is unable to normally produce flowers.

The condition is also known as phyllomorphy or frondescence; though the latter may sometimes refer more generically to foliage, leafiness, or the process of leaf growth. Phyllody is usually differentiated from floral virescence, wherein the flowers merely turn green in color, but otherwise retain their normal structure. However, floral virescence and phyllody (along with witch's broom and other growth abnormalities), commonly occur together as symptoms of the same diseases. The term chloranthy is also often used for phyllody (particularly flowers exhibiting complete phyllody, such that it resembles leaf buds more than flowers), though in some cases it may refer to floral virescence.

Symptoms

- All floral parts are transformed into green leafy structures followed by abundant vein clearing in different flower parts.
- In severe infection, the entire inflorescences is replaced by short twisted leaves closely arranged on a stem with short internodes, abundant abnormal branches bend down.
- Finally, plants look like witches broom.
- If capsules are formed on lower portion of plant they do not yield quality seeds.
- Transmitted by the vector *Orosius albicintus*

Mango Malformation

Introduction:

Mango (*Mangifera indica* L.) occupies a pre-eminent place amongst fruit crops in India and is acknowledged as ‘King of fruits’ in the country. Malformation is the most threatening malady that causes great economic loss and limits the mango production in India and among tropical and subtropical countries around the globe. Floral malformation, in contrast to vegetative one, is very virulent and can cause the loss of the entire crop. Affected panicles either do not set fruit or abort fruit shortly after they have set; yields can be reduced by as much as 50-80%. Mango Malformation Disease is a fungal disease of mangoes caused

by *Fusarium* species *Fusarium moniliforme* var. *subglutinans*. Mango is the only known host of the disease. Numerous studies on physiological, fungal, acarological, nutritional aspects have attempted, still the nature of the disorder is not fully understood.

Vegetative Malformation (VM): Vegetative Malformation (VM) is more commonly found on young seedlings. The seedlings produce small shootlets bearing small scaly leaves with a bunch like appearance on the shoot apex. Hence, the apical dominance is lost and the seedling remains stunted and numerous vegetative buds sprout producing hypertrophied growth, which constitutes vegetative malformation. The multi-branching of shoot apex with scaly leaves is known as "Bunchy Top", also referred to as "Witche's Broom". The seedlings, which become malformed early, remain stunted and die while; those getting infected later resume normal growth above the malformed areas.

Floral Malformation (FM): Floral Malformation (FM) is the malformation of panicles. The primary, secondary and tertiary rachises become short, thickened and hypertrophied. Such panicles are greener and heavier with increased crowded branching. These panicles have numerous flowers that remain unopened and are predominantly male and rarely bisexual. The ovary of malformed bisexual flowers is exceptionally enlarged and non-functional with poor pollen viability. Both healthy and malformed flowers appear on the same panicle or on the same shoot. The severity of malformation may vary on the same shoot from light to medium or heavy malformation of panicles. The heavily malformed panicles are compact and overcrowded due to larger flowers. They continue to grow and remain as black masses of dry tissue during summer but some of them continue to grow till the next season. They bear flowers after fruit set has taken place in normal panicles and contain brownish fluid.

On the basis of compactness of panicles, malformed panicles are classified into different group's viz., heavy, medium and light; compact malformed panicle, elongated malformed panicle and slight malformed panicle and small compact type and loose type. The panicles of heavy

type are very compact due to excessive crowding of flowers, keep growing to form large hanging masses of flowers, most of these dry up, and hang as brown discolored bunches, but some continue to grow till the next season. The medium types of malformed panicles are slightly less compact and persist on plant for a longer time than the normal panicles. The light type is only slightly more compact than the normal panicle and does not persist on the plant. Sometimes, a shoot tip may bear both types of panicles i.e., healthy as well as malformed. Less frequently, a healthy panicle may contain one or more malformed branches of a few malformed flowers or vice-versa. These partially infected panicles may bear fruits up to maturity.

1.4 PREPARATION OF DIFFERENT MEDIA FOR THE ISOLATION AND CULTURE OF FUNGI FROM SOIL AND DISEASED MATERIALS

Aim:

To prepare different media for isolation and culture of fungi

Principle:

The mycologist raises pure culture of a fungus to study the fungus in detail regarding its reproduction, physiology and genetics. The pure culture may be on liquid solid/media and in obtaining the pure culture of the organism, the mycologist employs certain procedure.

1. **Preparation of Culture-Media:**

Cleaning of Glass wares:

All glass wares used in microbiological or pathological studies should be rigidly cleaned. Initial cleaning should be done with soap or vim using a brush.

They can finally be washed with the following mixture:

Pottassium dichromate – 100 gms.

Conc. H_2So_4 – 500c.c

Distilled Water – 1000 ml

The same mixture can be used several times. After washing with this mixture, the glasswares are washed for sometimes in running tap water, then finally with distilled water and allowed to air dry.

Procedure:

Preparation of Media:

The choice of medium depends on the type of microorganism to be grown taking into consideration the nutritional requirements of the particular form.

Out of a large number of such media, some are described below:

(i) Based on the consistency, the culture media are of two types:

(a) Liquid media or Broth:

These media are liquid in consistency and are used in cases where microbial growth in pure form is to be separated out for further studies.

(b) Solid Media:

The liquid media can be solidified by the addition of Agar-Agar, a jelly like substance extracted from the sea-weed, Gelidium. Addition of about 1.5% of this substance at or near the neutral pH is sufficient to make the medium solid.

(ii) Based on the composition, the culture media may be of two types:

(a) Natural Media:

These media incorporate certain naturally available substances of **"Somewhat"** unknown composition. Some examples of such media are: Malt extract agar. Soil extract agar. Oat meal agar and Potato dextrose agar.

Potato dextrose agar is commonly used for phytopathological studies.

The composition of Potato Dextrose agar (PDA) is given below:

Potatoes (Peeled and Sliced)	—	200 gms.
Dextrose	—	20 gms.
Agar-Agar Powder	—	15-20 gms.
Distilled water	—	1000 ml.

Potato slices are allowed to simmer in 500 ml. distilled water, the extract filtered by means of a muslin cloth, dextrose and agar-agar powder are added, the volume made up to 1000 ml and made to boil.

(b) Synthetic Media:

These media have known constituents.

Examples of synthetic media are Standard synthetic and Czapek-Dox media, composition of which is given below:

Standard synthetic Agar (Brown's medium)

$MgSo_4$. $7H_2O$	-	0.5 gms.
K_2H PO_4	-	0.5 gms.
KNO_3	-	2.0 gms.
Starch Soluble	-	20.0 gms.
Agar Agar	-	15 gms.
Distilled Water	–	1000 ml

Czapek Dox Agar

NaNo3	**-**	**2.0 gms.**
KH_2 PO_4	-	1.0 gms.
KCL	**-**	**0.5 gms.**
$MgSo_4$. $7H_2O$	-	0.5 gms.
$FeSo_4$. $7H_2O$	-	0.01 gms.
Sucrose	-	30 gms.
Agar – Agar	-	15 gms.
Distilled Water	-	1000 ml

All the constituents are weighed out distilled water added and the mixture heated to boiling. It will be better to add K_2HPO_4 or KH_2PO_4 in the end to avoid precipitation. The pH is adjusted as desired by adding NaOH or Citric acid solutions in required quantity.

Filtration of Media and dispensing into culture tubes or Flask:

To a large funnel, a rubber tubing with a stop-cock is fitted. The medium is filtered through a piece of muslin cloth and the appropriate quantities are dispensed into culture tubes (6″x 3/4″) or flasks (250 ml capacity), by adjusting the stop-cock.

All the glasswares in which the medium it is to be dispensed must be plugged with cotton wool. Culture tubes meant to be agar slants should receive approximately 10 ml of the medium while those ultimately to be used for plating (in flasks) approximately 50ml. The

remaining medium should be put in flasks, plugged with cotton wool and used as required.

Isolation of Fungi: (Streptomycin-peptone-dextrose Medium of Johnson 1957)

Composition:

KH_2PO_4	–	1.0 gm.
$MgSO_4$, $7H_2O$	–	0.5 gm.
Peptone	–	5.0 gm.
Dextrose	–	10.0 gm.
Distilled Water	–	1000 ml.
Rose Bengal	–	10.0 ml. (1:30,000)
Streptomycin	–	30 µg/ml.
or Aureomycin	–	20 µg/ml.

20 gms. of agar is dissolved in 250 ml. of distilled water in a flask by heating on a water-bath. All the other ingredients except Rose-Bengal and antibiotics are dissolved in 750 ml. water in a flask and then this solution is poured in the agar solution.

The mixture is then heated and stirred continuously, till it boils. After removing from the heater 10 ml. of Rose-Bengal (1:30,000) dilution is added at the rate of 1 ml/100 ml. of medium.

The medium is then dispensed in 100 ml. conical flasks at the rate of 100 ml. per flask. The flasks are then plugged and autoclaved at 15 lbs. pressure for 20 minutes. The antibiotic solution (streptomycin) is added before plating the medium at nearly 45°C (streptomycin inhibits the growth of bacteria while Rose-Bengal is a growth retardant).

4.1 Isolation of fungal pathogen from diseased plant material:

Aim:

To isolate fungal pathogen from the given plant material.

Principle

Inspection of dry seed can be applied to detect seed- borne pathogen which when present in the seed may cause discoloration of seed coat or changes in the seed size and shap**e.** The inspection of dry seed in seed health testing is a qualitative test for which no standard working

sample size has been worked out. However, it may be suggested to use a sample equal in size to the sample employed in the purity analysis in seed certification.

Examination of Seeds without Incubation

Dry seed Examination

The seed sample is first examined by naked eyes, then under stereoscopic binocular microscope to record observation on the mixture of seeds, weed seeds, plant parts, inert matter, discoloration, malformations, sclerotia, galls, bunt balls, bacterial ooze, fungal bodies like, acervuli, Pycnidia, Perithecia, hyphae, spore masses etc. Mechanical damage of seed is also recorded as they act suitable site for the entry of pathogen.

According to the rules of the International seed testing Association (ISTA), the inert matter fraction such as soil, sand and stones, various types of plant debris, including nematode galls, fungal bodies such as ergot sclerotia and smut balls, are of great pathological importance.

All parts of a seed sample are examined carefully by naked eye or with the help of hand lens. During the examination, emphasis is laid on galls, sclerotia and smut balls; the technique is simple and gives quick information about the health status of the seed lot.

Procedure:

a) Take 400 seeds at random in a Petri-plate.

b) Note down the weight of the seeds.

c) Examine the seed samples with the help of stereoscopic binocular microscope.

d) Make suitable record out of 400 seed.

(ii) Washing Test:

The washing test is a seed health testing method which is used solely to test seeds for externally seed borne pathogens, the inoculum of which is present loosely on the seed surface.

The washing test is a qualitative test for which no standard working sample has been approved so far by the ISTA. The washing test as

mentioned here is generally used to detect presence or absence of fungal prop gules.

Procedure:

a) Two gm of seed is taken in a test tube with 10 ml of water and shaken for 10 minutes on a mechanical shaker.

b) The suspension is examined as such or the suspended spores are concentrated by centrifuging at 3000 rpm for 15-20 minutes.

c) The suspension is discarded and the spores are again suspended in 2 ml of lacto phenol (a mixture of lactic acid, phenol, water and glycerol in the ratio of 1:1:1:2).

d) This suspension is then examined under the microscope for the presence of spores, conidia and other fructifications.

e) Rice (1939) suggested the use of haemocytometer for the semi-quantitative estimation, where the spores load/gm of seed can be calculated with the help of the following formula:

$$\frac{N \times v}{0.0001} = N \times V \times 10{,}000$$

(0.0001 being the value of fluid in Central Square of the haemocytometer)

Where, N is the number of spores in the central square.

V is the value of mounting fluid added to the sediment

W is the weight of seeds

So, the spore's load/g of seed will be:

$$\frac{N \times V \times 10{,}000}{W}$$

(iii) Viability Test:

To test the viability of the spore obtained from the washing test, the suspension containing spores is spread on 2 per cent plain agar. The viability can also be checked by streaking the spore's suspension on potato dextrose agar medium. Incubate the Petriplates containing plain agar or PDA for 5 days at 25 ± 1°C (Fox 1993).

1.5 ISOLATION OF BACTERIA FROM DISEASED PLANT SPECIMEN

Aim:

To isolate bacteria from the diseased plant specimen and to study its features, symptoms and occurrence.

Principle:

The term 'pathogen' means the organism that incites diseases on living being. It may be fungus, bacteria, virus etc. The pathogen can easily be isolated in artificial culture media for identification and subsequent characterization.

Requirements:

1. Infected plant parts/plant
2. Petridishes
3. Forceps and knife
4. Mercuric chloride solution (1:1000)
5. Distilled water (steriled)
6. Slants with desirable culture media
7. Incubator
8. Bunsen burner
9. Slide and cover glass
10. Microscope
11. Absolute alcohol

Procedure:

(1) An infected plant or leaf of rice was brought to the laboratory from the field for isolation of pathogen.

(2) Initially the symptoms were examined under microscope or by hand lens. The infected lesionic part was removed by a knife and quickly transferred to the sterilising solution.

(3) Surface sterilisation of the infected plant was done by transferring the excised infected leaf segment into a petridish containing mercuric chloride solution (1: 1000) and kept for 2 – 3 min.

(4) Then the leaf segment was transferred to a series of petridishes containing sterile distilled water for washing of mercuric chloride.

(5) Finally the excised leaf segment was placed aseptically into the slant for culture of pathogen.

(6) The slant was then incubated at a required temperature for 3 – 5 days after proper leveling.

(7) Finally, the culture thus developed was examined microscopically.

Preparation of Media for Isolation of Bacteria:

Media Composition:

K_2	–	0.4 gm.
NH_4HPO_4	–	0.5 gm.
$MgSO_4.7H_2O$	–	0.05 gm.
$MgCl_2$	–	0.1 gm.
$CaCl_2$	–	0.1 gm.
$FeCl_3$	–	0.01 gm.
Peptone	–	1.0 gm.
Yeast extract	–	1.0 gm.
Agar agar	–	20 gm.
Distilled Water	–	750 ml.
pH	–	7.4

20 gms. of agar is weighed, taken in a flask containing 500 ml distilled water and heated in a water-bath until the agar melts. In another flask all the other ingredients are taken along with 250ml. of water. This solution is added to the melted agar solution and the pH of the medium is adjusted to 7.4.

The medium is then dispensed in tubes, each containing about 18 c.c. of the medium. The tubes are then plugged and autoclaved at 15 lbs. pressure for 20 min.

(b) Inoculation and Plating:

(i) Incorporation:

The stabs containing different isolation media are heated in a water-bath for uniform melting. These are cooled to nearly 45°C and 1 ml. of soil suspension of definite dilution is poured in each of the tubes by a pipette. Each tube is rolled in a vortex mixer for thorough mixing.

The medium with soil suspension of each tube is plated in sterilised petridishes aseptically and allowed to solidify. After solidification, the petridishes are incubated at 30°C in an incubator inversely except petridishes for algae isolation which are placed in an open air lighted environment keeping them straight (upper lid upward). Soil suspension of 10^{-6} and 10^{-7} dilution are used for isolating actinomycetes and bacteria; 10^{-4} and 10^{-5} for fungi and 10^{-2} and 10^{-3} for algae.

(ii) Spreading:

Some selected media are inoculated in this technique. In this method, the media contained in, the flasks are melted and antibiotic added after cooling to 45°C. After thorough mixing, these are plated in sterilised petridishes and allowed to solidify.

After solidification, 0.2 ml. of soil suspension of 10^{-4} and 10^{-5} dilution are added separately to the medium by a pipette and spread out by a glass spreader. The whole thing is done aseptically. Then they are incubated invertedly at 30°C or at 27°C.

(c) Plate Reading:

After 48 hrs. and 96 hrs. The plates are taken out from incubator, the number of colonies in each plate is counted by a colony counter and at the same time the nature of the colonies is observed with the naked eye (Fig.3.1).

(d) Determination of Water Content of Soil Sample:

10 gms. of soil sample is taken in a clean petridish and kept in a hot air oven at 110°C for 3 – 5 hrs. and then kept at 60°C overnight. On the next day the weight of the soil is taken and thus the dry weight is found. Loss of weight is due to evaporation of water from the soil.

1.6 COLLECTION AND STUDY OF THE CROP DISEASES FROM THE LOCAL AND OUT STATION FIELDS

Aim:

To collect and study crop diseases from the given sample.

Introduction:

Plant pathogens:

Plant pathogens can be fungal, bacterial, viral or nematodes and can damage plant parts above or below the ground. Identifying symptoms and knowing when and how to effectively control diseases is an ongoing challenge for growers of Tomato wilt, Citrus canker, Loose Smut of Wheat.

In general, a plant becomes diseased when it is continuously disturbed by some causal agent that results in an abnormal physiological process that disrupts the plant's normal structure, growth, function, or other activities. This interference with one or more of a plant's essential physiological or biochemical systems elicits characteristic pathological conditions or symptoms.

Plant diseases can be broadly classified according to the nature of their primary causal agent, either infectious or noninfectious. Infectious plant diseases are caused by a pathogenic organism such as a fungus, bacterium, mycoplasma, virus, viroid, nematode, or parasitic flowering plant. An infectious agent is capable of reproducing within or on its host and spreading from one susceptible host to another. Noninfectious plant diseases are caused by unfavorable growing conditions, including extremes of temperature, disadvantageous relationships between moisture and oxygen, toxic substances in the soil or atmosphere, and an excess or deficiency of an essential mineral. Because noninfectious causal agents are not organisms capable of reproducing within a host, they are not transmissible.

In nature, plants may be affected by more than one disease causing agent at a time. A plant that must contend with a nutrient deficiency or an imbalance between soil moisture and oxygen is often more susceptible to infection by a pathogen, and a plant infected by one pathogen is often prone to invasion by secondary pathogens. The combinations of all disease-causing agents that affect a plant make up the disease complex. Knowledge of normal growth habits, varietal characteristics, and normal variability of plants within a species as these

NOTES

relate to the conditions under which the plants are growing is required for a disease to be recognized.

6.1 Tomato Wilt Caused *Fusarium oxysporum*:

Fusarium wilt of tomato is a soilborne disease that occurs worldwide. Once introduced to a field, the *Fusarium* wilt pathogen is almost impossible to eradicate. The best way to manage *Fusarium* wilt is to plant tomato varieties that are resistant to the races of the pathogen that are present in the field. *Fusarium* wilt of tomato occurs in most regions where tomatoes are grown. This disease can result in yield losses of up to 80% when severe.[1,2] The pathogen can infect the crop at all growth stages, entering through the roots. The fungus grows into the xylem (water conducting tissue) where it can spread within the plant. Colonizing the xylem results in the inhibition of water flow and the wilt symptoms.

Pathogen:

Fusarium wilt is caused by the fungus *Fusarium oxysporum* forma specialis *lycopersici* (*Fol*). The "forma specialis" designation means that this specialized version of the species infects tomatoes. Three races (1,2, and 3) of the pathogen have been identified based on their ability to cause disease on tomato varieties with different forms of disease resistance.

Race 1 infects varieties with no genetic resistance to *Fusarium* wilt. This race is widely distributed, found in most tomato growing areas of the world. Race 2 was first identified in 1945 from a tomato variety that is resistant to race 1. Race 2 can now be found in many tomato growing areas of the world. Race 3 was first identified in Australia in 1978, and a few years later it was detected in Florida and then in California in 1987 on tomato varieties that are resistant to both races 1 and 2. Research indicates that the race 3 isolates have developed locally from race 2 isolates in California and Florida, rather than being introduced from Australia. In California, race 3 was found only in the Sutter Basin area for several years, but it has now spread to Fresno

County and other major processing tomato growing areas in the state. Race 3 is now present in Mexico and areas of North and South Carolina.

Symptoms

The initial symptoms of *Fusarium* wilt are a yellowing and wilting of leaves, usually after flowering when the tomato fruit are starting to increase in size. These symptoms often develop only on one side of a plant, or on one branch, or even on one side of a leaf. This pattern of symptom expression distinguishes *Fusarium* wilt from other wilt diseases of tomato. The symptoms start on the lower leaves of the plant and move upward as the disease progresses. At first, the leaves wilt during the warmest part of the day but may recover overnight. Eventually, the entire plant turns yellow, wilts, and dies. Infected plants are often stunted and produce fewer fruit.

6.2. Citrus Canker

Introduction to the Citrus Canker Disease:

Citrus Canker is a bacterial disease of worldwide distribution occurring wherever citrus is grown. It is a serious menace to our most valued citrus orchards causing objectionable blemishes on the fruit. The disease causes serious damage in India, China, Japan and Java.

The pathogen incites severe canker disease in a number of citrus species on stems, leaves and fruits. The disease attacks most of the species/varieties of citrus. The most susceptible species are the acid lime plants, the sweet orange and the grape fruit.

Causal Organism:

The causal organism is the bacterial pathogen *Xanthomonas citri*, now called *X. campestris* pv. citri (Hasse) Dowson. It consists of a short, motile rod (1.5-2.0 x 0.5- 0.75 µ) furnished with a single polar flagellum (monotrichous). It lacks endospore formation. It is a gram negative, aerobic form surrounded by a mucilaginous capsule. It forms chains.

The climate factors which favour the disease are the mild temperature and wet weather. The most suitable range of temperature appears to be 20ºC to 30ºC.

Symptoms of Citrus Canker Disease:

Crast-like disease lesions or scabby spots and small cankers (open wounds or dead tissue surrounded by living tissue) appear on all over ground parts of the plant such as leaves, young branches and fruits. The trees are, however, not commonly killed.

The lesions on the foliage, at first, appear on the lower surface as small round raised spots. These are translucent and of yellowish brown colour. Later the spots turn white or greyish and finally rupture. The older lesions are corky and brown, sometimes purplish.

The necrotic brownish canker regions are surrounded by a yellowish brown to green raised margin and distinct watery yellow halo region. The yellow halo region is free from the pathogen. The cankerous lesions contain the pathogen in millions.

Mairie suggested that the halo regions are formed due to the response of the host tissue to a diffusible metabolite of the pathogen. Padmanabhan et al. (1975) reported accumulation of malic acid in the halo region due to increased respiration in this region.

The lesions on the twigs are usually irregular in form. The lesions on the fruit are similar to those on the leaves but lack the yellow halo.

6.3. Loose Smut of Wheat:

Introduction:

Loose smut of wheat is a destructive disease. The disease was recognised and confirmed very early than the other wheat diseases. Maddox (1895) was the first to show the nature of the disease. Due to the characteristic black dusty appearance of diseased inflorescence, it is also called as smut, black smut, black head, blasted head and snuffy ear.

The disease is available throughout the world and is more abundant in humid and semi- humid regions, cause great loss in yield. In India the loss is much more in cooler and moist northern region than the south.

Mishra and Singh (1969) reported 3-6% loss in some newly developed high yielding varieties of wheat. Later, Gothwal et at., (1972) observed that smutted plant produce 18.7% reduction in height of plants,

22.9% less tillers, 27.5% shorter peduncles and 15.7% reduction in yield.cEarlier, Luthra (1953) estimated an annual loss of fifty million rupees only in Punjab.

Causal Organism:

The pathogen is *Ustilago segetum* (Pers.) Roussel var. tritici (earlier was known as *Ustilago tritici* (Pers.) Rostr.).

The black powdery mass of spores, the chlamydospores, are olive-green, spherical to oval in shape, minutely echinulate walls and 5-9 µm in diameter. The chlamydospore germinates to develop a promycelium of four uni-nucleate cells (basidium) and each cell later develops into germ tube or infection thread.

No sporidia are developed from uninucleate cells of promycelium. The spores remain viable for 5-6 months. The fungus is unable to survive saprophytically in the soil.

Host:

Triticum aestivum L.

Symptoms:

The symptom is visible only when the plants develop ears. The ears of the affected plants emerge out of the flag leaf earlier than the healthy ones. In infected plants, generally all the ears and all the spikelets and kernels of each ear are smutted. The spikelets of the affected ear get deformed and filled with dry, black, powdery mass of spores, thus no grains are formed.

Initially the spores are covered by a delicate greyish membrane which soon burst and the spores get free. The spores are blown off by wind leaving only the naked rachis. Rarely some of the tillers developed from infected seed may escape from the disease. The number and length of the tiller is reduced in infected plant. The growth and general appearance of the plant is not affected. The seeds become infected without showing any change in the external morphology.

1.7 DEMONSTRATION AND PRODUCTION OF CELL WALL DEGRADING ENZYMES

Aim

To demonstrate the production of cell wall degrading enzymes.

Principle:

Plant cell wall-degrading enzymes (PCWDEs) play significant roles throughout the fungal life including acquisition of nutrients and decomposition of plant cell walls. Particularly for plant pathogens, it is critical to decide where and when to start intruding into the host cell. Many plant pathogens are known to secrete a variety of PCWDEs to perceive weak regions of plant epidermal cells and penetrate the plant primary cell wall. For example, a cutinase (CUT2) in the rice blast fungus, *Magnaporhte oryzae*, is known to play roles in hydrophobic surface sensing, differentiation and virulence on rice and barley.

Procedure:

Enzymatic Method:

Enzymatic method is a very widely used technique for the isolation of protoplasts. The advantages of enzymatic method include good yield of viable cells, and minimal or no damage to the protoplasts.

Sources of protoplasts:

Protoplasts can be isolated from a wide variety of tissues and organs that include leaves, roots, shoot apices, fruits, embryos and microspores. Among these, the mesophyll tissue of fully expanded leaves of young plants or new shoots is most frequently used. In addition, callus and suspension cultures also serve as good sources for protoplast isolation.

Enzymes for protoplast isolation:

The enzymes that can digest the cell walls are required for protoplast isolation. Chemically, the plant cell wall is mainly composed of cellulose, hemicellulose and pectin which can be respectively degraded by the enzymes cellulose, hemicellulose and pectinase. The different enzymes for protoplast isolation and the corresponding sources are given in Table 44.1.

In fact, the various enzymes for protoplast isolation are commercially available. The enzymes are usually used at a pH 4.5 to 6.0, temperature 25-30°C with a wide variation in incubation period that may range from half an hour to 20 hours.

The enzymatic isolation of protoplasts can be carried out by two approaches:

1. Two step or sequential method:

The tissue is first treated with pectinase (macerozyme) to separate cells by degrading middle lamella. These free cells are then exposed to cellulose to release protoplasts. Pectinase breaks up the cell aggregates into individual cells while cellulose removes the cell wall proper.

2. One step or simultaneous method:

This is the preferred method for protoplast isolation. It involves the simultaneous use of both the enzymes — macerozyme and cellulose.

Isolation of protoplasts from leaves:

Leaves are most commonly used, for protoplast isolation, since it is possible to isolate uniform cells in large numbers.

The procedure broadly involves the following steps:

1. Sterilization of leaves.
2. Removal of epidermal cell layer.
3. Treatment with enzymes.
4. Isolation of protoplasts.

Besides leaves, callus cultures and cell suspension cultures can also be used for the isolation of protoplasts. For this purpose, young and actively growing cells are preferred.

Purification of protoplasts:

The enzyme digested plant cells, besides protoplasts contain undigested cells, broken protoplasts and undigested tissues. The cell clumps and undigested tissues can be removed by filtration. This is followed by centrifugation and washings of the protoplasts. After centrifugation, the protoplasts are recovered above Percoll.

Viability of protoplasts:

It is essential to ensure that the isolated protoplasts are healthy and viable so that they are capable of undergoing sustained cell divisions and regeneration.

1.8 DEMONSTRATION OF THE PRODUCTION OF MYCOTOXINS

AIM:

To demonstrate the production of mycotoxins

Principle:

A mycotoxin is a toxic secondary metabolite produced by organisms of the fungus kingdom and is capable of causing disease and death in both humans and other animals. The term 'mycotoxin' is usually reserved for the toxic chemical products produced by fungi that readily colonize crops.

Introduction:

The mycotoxins are produced by more than 100 mould species, mainly by Aspergillus, Penicillium and *Fusarium*. According to World Health Organisation (WHO), 1969, the mycotoxins associated with human diseases are of four types. These are aflatoxins, ochratoxins, zearalenone, and trichothecenes.

(a) Aflatoxins:

Aflatoxins are potent toxic, carcinogenic, mutagenic, immuno-suppressive agents, produced as secondary metabolites by the fungi on variety of food products. The anatoxin problem was first recognised in 1960, when there was severe outbreak of a disease referred as "Turkey X' Disease" in U.K., in which over 100,000 turkey birds died.

Aflatoxins are the most potent carcinogens, known as the suspected cause of liver cancer in human beings. The aflatoxins are produced in dried food and groundnut meal, infested with Aspergillus flavus, A. parasiticus, A. fumigatus or Penicillum islandicum.

Aflatoxins normally refers to the group of difuranocoumarins and are classified in two broad groups according to their chemical structure the difurocoumarocyclopentenone series and the difurocoumarolactone series. About 18 types of aflatoxins have been identified, the major

members are aflatoxin B_1, B_2, G_1, G_2, while M_1, and M_2 are less significant.

Aflatoxins are soluble in methanol, chloroform, acetone, acetonitrile and fluoresce strongly in ultraviolet light (365 nm); B_1 and B_2 produce a blue fluorescence, while G_1 and G_2 produce green fluorescence.

Aflatoxins bind with DNA to prevent transcription, thereby protein synthesis is inhibited.

In addition to suspected carcinogenic nature, other clinical symptoms due to aflatoxin poisoning are jaundice, portal hypertension, rapidly developing ascites, etc.

(b) Ochratoxins:

Ochratoxins are closely related derivatives of isocoumarin, linked to an amino acid, L-β-phenylalanine. Of the nine types of ochratoxins, ochratoxin A is the most effective one. Ochratoxins are produced in maize, peanuts, and beans etc., infested mainly with *Penicillium viridicatum* or *Aspergillus ochraceus*.

(c) Zearalenone:

Zearalenone is a phenolic resorcyclic acid lactone, produced in maize and also in other cereals, infested with different members of *Fusarium* such as F. moniliformae and F. graminearum. It shows esterogenic symptoms in swine.

(d) Trichothecenes:

Trichothecenes possess a tetracyclic 12, 13-epoxytrichothecoene skeleton. So far, more than 30 trichothecenes have been identified, of which T-2 toxin, nivalenol and deoxynivalenol, shows marked importance.

Trichothecenes are produced by different species belongs to the genera *Fusarium, Myrothecium, Trichoderma, Stachybotrys* and *Cephalosporium*. This group of toxins shows sub-epidermal haemorrhage, several local irritations and general necrosis.

1. Mycotoxins of Aspergillus:

The Aflatoxins. In 1959 a very singular event occurred which initiated the international interest which now exists in mycotoxins.

This was the deaths of several thousand turkey poults and other poultry on farms in East Anglia and, because of the implications for the turkey industry and the manufacture of pelletted feed which supported it, a considerable effort was put into understanding the etiology of this major outbreak of what was initially referred to as turkey X disease.

Although the name implies a disease such as a viral infection, it was shown that the birds had been poisoned by a contaminant in the groundnut meal used as a protein supplement in the pelletted feed. The contaminant, which was called aflatoxin, fluoresces intensely under ultra-violet light and was shown to be produced by the mould Aspergillus flavus growing on the groundnuts.

Aflatoxin is not only acutely toxic but, for the rat, it is amongst the most carcinogenic compounds known. The demonstration of the potential carcinogenicity of aflatoxin made it possible to rationalize the etiology of diseases such as liver carcinoma in rainbow trout and hepatitis X in dogs which had been described nearly a decade earlier but had remained a mystery.

Very sensitive analytical methods for aflatoxins were developed which led to the demonstration that their occurrence was widespread in many agricultural commodities, especially groundnuts and maize, much of which may be destined for human consumption.

2. Mycotoxins of Penicillium:

Penicillium is much more common as a spoilage mould in Europe than *Aspergillus* with species such as *P. italicum* and *P. digitatum* causing blue and green mould respectively of oranges, lemons and grapefruits, *P. expansum* causing a soft rot of apples, and several other species associated with the moulding of jams, bread and cakes.

Species which have a long association with mould-ripened foods include *P. roquefortii* and *P. camembertii*, used in the mould ripened blue and soft cheeses respectively.

The mycotoxin patulin is produced by several species of *Penicillium*, Aspergillus and Byssochlamys but is especially associated with *P. expansum* and was first described in 1942 as a potentially useful antibiotic with a wide spectrum of antimicrobial activity.

3. Mycotoxins of *Fusarium*:

Some species of *Fusarium* cause economically devastating diseases of crop plants such as wilts, blights, root rots and cankers, and may also be involved in the post-harvest spoilage of crops in storage. The genus is also associated with the production of a large number of chemically diverse mycotoxins.

1.9 CULTURE AND STUDY OF SOME COMMON PLANT PATHOGENIC FUNGI

Aim:

To study common pathogenic fungi

Introduction:

Fungi are some of the most important organisms in the world. They play vital roles in ecosystem functioning and also have a considerable influence on humans and human-related activities. Plant pathogens are among the most important of all fungi, since they have profound negative influences in many different types of ecosystems. For example, some plant pathogens cause huge losses in crop and tree production and spoilage of commodities in post-harvest storage; others, such as *Fusarium oxysporum* and *Aspergillus* in India, may impact negatively on natural ecosystems.

1. *Fusarium*

Habit and Habitat of *Fusarium*:

Fusarium is represented by large number of species which occur both in temperate and tropical regions of the world. Majority of the species are saprophytic, some are mild facultative parasite while some species are parasitic and cause serious diseases like wilt (Fig. 1) or 'rot' of economically important plants.

Fig. 1. *Fusarium.* Wilt of *Cajanus cajan* (arhar)

Vegetative Structure of *Fusarium*:

Mycelium is branched, sepate, hyaline or coloured, inter-or intracellular and uninucleate to multinucleate. Hyphae invade the tracheids and vessels of xylem, ramify there, produce toxic substances and block them completely. As a result the plants wilt and die.

3. Reproduction of *Fusarium*:

It takes place by the formation of three kinds of asexual spores. These are:

(a) Micro conidia,

(b) Macro conidia, and

(c) Chlamydospores.

(a) Micro Conidia:

The micro conidia and macro conidia are produced in same sporodochia. These sporodochia develop on the surface of stem, leaves and other parts of the host plant. The fungal mycelium collects near the surface of the host tissue as anpseudoparenchymatous mass. It gets exposed by the rupturing of the epidermis (Fig. 2).

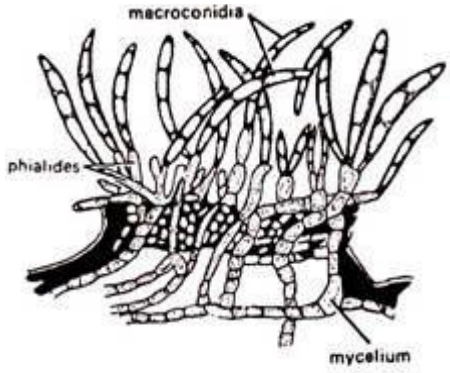


Fig. 2. Fusarium : Sporodochium.

From the fungal hyphae arise many short and cylindrical structures. These are conidiophores. The ultimate branches of conidophores which produce conidia are called phialides. The phialides are subulate i.e. owl shaped and have some kind of heel (characteristic of *Fusarium*, Fig. 3).

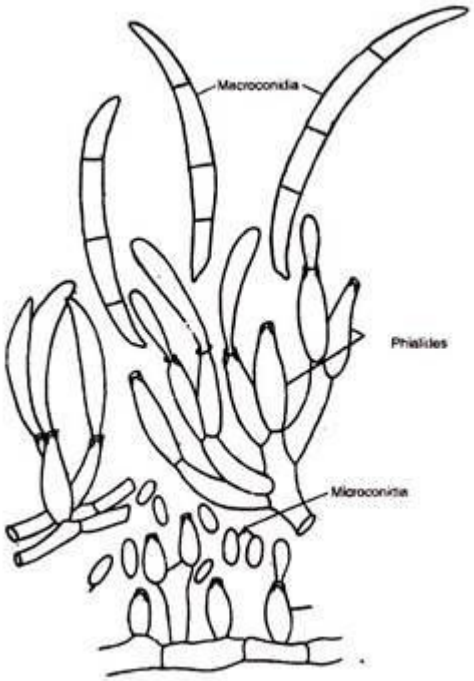


Fig. 3. Fusarium : Phialide, microconidia and macroconidia.

Micro conidia are small, unicellular or bi-celled, spherial or oval in shape. Their size varies from 5×15 μ x2−4 μ. They are borne single or in chains on the conidiophores by abstraction method.

(b) Macro Conidia:

The macro conidia are long, sickle or crescent shaped, multi-septate (3-5 septa), pointed at the end and broad in the middle.

They measure 15-50 µ in length and 3-5 µ in breadth. Both macro conidia and micro conidia are produced in vast numbers. They are easily disseminated by wind and after falling on the suitable substratum, they germinate and infect the host plants.

(c) Chlamydospores:

Under relatively starvation and dry conditions, the mycelial hyphae produce ovoid or spherical thick walled cells. These are called chlamydospores. They occur either single or in chains and may be terminal or intercalary (Fig. 4) in position. After maturity they get separated from the parent hyphae and act as resting spores, under favourable conditions they germinate by means of germ tubes to form a fresh mycelium.

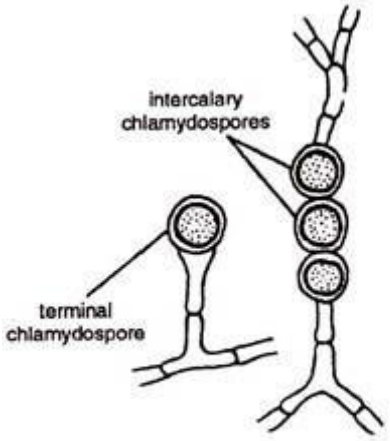


Fig. 4. *Fusarium* : Chlamydospores

The pseudoparenchymatous mycelium of *Fusarium* often forms black, compact bodies known as sclerotia. They act as storage organ and also serve as means of perennation and vegetative reproduction.

Culture Media:

The four media used for growing *Fusarium* species for identification are carnation leaf agar, potato dextrose agar, KCl medium, and soil agar. Carnation leaf agar has the advantage of promoting sporulation rather than mycelial growth. Conidia and conidiophores of most species are produced in abundance, their morphology closely approximates that seen under natural conditions, and phenotypic variation is reduced. The value of carnation leaf agar as a growth medium may be due to the facts that it is low in available carbohydrates and it contains

complex, naturally occurring substances of the type encountered by *Fusarium* species in nature. Therefore, the fungi grow and sporulate in a manner similar to that found on a host plant or natural substrate. Identification procedures can be based almost exclusively on cultures grown on this medium.

Potato dextrose agar is a valuable medium used principally for noting gross morphological appearances and colony colorations. Because of its high available carbohydrate content, potato dextrose agar generally emphasizes growth to the detriment of sporulation. Cultures grown on this medium sporulate poorly, frequently taking more than a month to do so. The conidia produced are often misshapen and atypical. Consequently, with few exceptions, potato dextrose agar cultures are not used for microscopic observations. Cultures grown on potato dextrose agar are used only in a secondary role. KCI medium is used to observe the formation of microconidia in chains by species in section Liseola. The species that do form chains of microconidia form more abundant, longer chains on this medium. The chains are easier to observe because there is less moisture on the surface of the agar and fewer droplets of moisture in the aerial mycelium.

Direct observation under a microscope of 4 - to 5 day old cultures in petri dishes will demonstrate whether or not chains of microconidia are formed and may reveal the presence of monophialides or polyphialides. Soil agar is helpful in promoting rapid chlamydospore formation in a number of *Fusarium* species. A large piece of inoculum from an actively growing culture is used to inoculate the soil agar in a petri dish. Chlamydospore formation occurs primarily in the original piece of inoculum, with very few forming in the soil agar itself. Cultures that require up to 30 days for chlamydospore formation on other media may form chlamydospores in 4 to 6 days on soil agar.

2. Aspergillus:

Introduction:

This fungus is also called Eurotium. It is chiefly a saprophytic fungus which is widely distributed. It grows on decaying vegetables; on

fatty media such as butter and ghee; on starchy media as bread and rice; on preserved fruits as jams and jellies. It is very commonly found on rotting oranges and phyllanthus fruits. It thus grows on a wide variety of substrata by virtue of the large number of enzymes it produces. All that the fungus requires are some organic matter and little of moisture. *Aspergillus* appears in the form of greenish, smoky patches along with *Mucor*, *Rhizopus* and *Penicillium* on moist bread when kept under a bell jar for a couple of days. The other common shades are yellow, black and blue. In all *Aspergillus* includes about 200 species. Majority of these are known only in the conidial stage. A few produce cleistothecia (perfect stage).

At first when the connection between the two stages was not fully established the sexual or perfect stage was called Eurotium and the conidial or imperfect stage as Aspergillus. Now both the stages are usually denoted by the common generic name Aspergillus because conidial stage is common and predominant and moreover generic name Aspergillus was introduced first. *Aspergillus* is a fungus whose spores are present in the air we breathe, but does not normally cause illness. In those people with a weakened immune system, damaged lungs or with allergies, *Aspergillus* can cause disease. Common *Aspergillus* infections include invasive aspergillosis, ABPA CPA and aspergilloma.

Aspergillosis is a group of diseases which can result from aspergillus infection and includes invasive aspergillosis, ABPA CPA and aspergilloma. Asthma is also complicated and exacerbated by *Aspergillus* infection (SAFS). Aspergillus affects humans and birds and animals can also develop aspergillosis, commercially many plant diseases and food spoilage may be due to aspergillus infection. Visit LIFE-Worldwide for detailed information on other types of fungal infections such as cryptococcus, candida and many others.

Aspergillus fumigatus is easily cultured and grows quickly. It is harder to get it to grow filamentously in liquid culture as it readily pellets.

Materials

- 260 ml vented tissue culture flasks
- Loops
- Glass beads
- PBS (Phosphate buffered Saline): make up using a PBS tablet, autoclave and add Tween 80 to 0.05 %.
- Potato dextrose agar / broth
- Sabourauds dextrose agar / broth
- Modified Vogel's minimal medium : Vogel's salts in 1% glucose
- Glycerol

Procedures

a) Growth on tissue culture plates

1) Pour 50 ml of either potato dextrose agar or Sabourauds dextrose agar into a 260 ml tissue culture plate and leave to set.

2) Place a loop of spores from a stock spore solution into the middle of a culture plate. Incubate at 30 to 37°C for 5-10 days with periodic checking. Examine microscopically for any sign of contamination.

3) Place a few glass beads and 8 ml of PBS into the flask and close the lid tightly. Shake the flask vigorously back and forth until most of the spores have been dislodged. Remove the spores using a pasteur pipette and store at 4°C. If necessary spores can be filtered through 2 layers of Whatman 105 lens tissue to remove hyphal fragments. A yield of 2-4 x 10^8/ml should be obtained.

b) Long term storage

1) Aliquot out 150 - 300 mµl glycerol into cryotubes and add 700 - 850 µl stock spore solution in PBS. Mix and store at - 80°C or in liquid nitrogen.

2)Stocks can also be maintained for months by plating stocks onto agar slopes in 5 ml bottles and storing the slopes at 4°C after the cultures have grown.

c) Overnight culture in liquid medium

1) Inoculate 50 ml of Vogel's minimal medium, Saubouraud dextrose broth or potato dextrose broth to a final concentration of 10^7 spores / ml

and incubate with shaking at 200 rpm until late exponential phase (18-24 h) at 37°C.

2) Dry down the mycelium onto Whatmann 54 paper using a Buckner funnel and a side-arm flask attached to a vacuum pump and wash with 0.6 M $MgSO_4$.

3. To freeze dry, transfer the mycelium to a universal tube, freeze at -80°C for a few hours, loosen the lid and freeze dry overnight. Seal the tube with parafilm and store at room temperature.

Tips and general comments

1) We grow *A.fumigatus* on solid medium in tissue culture flasks rather than on standard microbiological plates for 3 reasons: a) to control the spread of spores; spore cross-contamination is less likely with narrow-necked flasks b) the large surface area also means that fewer flasks are required to give a good yield of spores c) it is easier to harvest spores without creating an aerosol.

2) If vented flasks are not used, make sure the lids are loose and sealed with parafilm to enable aerobic growth. This will facilitate conidiation.

3) To limit pellet formation during liquid culture, we suggest using a high spore inoculum and disrupting the flow of the liquid by placing the flasks at an angle to the horizontal.

4) Spore viability is reduced when storage is at -80°C because of temperature fluctuations in freezers. It is preferable to store stocks in a liquid nitrogen ewer.

5) If spores in PBS are to be kept at 4°C for a few weeks, then a sensible precaution would be to add penicillin and streptomycin to final concentrations of 100 IU/ml and 100µg/ml respectively and to keep a close watch for contamination.

FGSC link: Media for culture of *Aspergillus nidulans*

References

Vogel, H.J. (1956) A convenient growth medium for Neurospora (medium N). Microbiol. Gen. Bull. 13, 42 – 44

2. Ecology, Biodiversity Conservation, Economic Botany

NOTES

2.1 STUDY OF MINIMAL SIZE OF THE QUADRATES BY SPECIES AREA CURVE METHOD IN FOREST AND GRASSLAND

Aim: To determine the minimum size of the quadrat by species area-curve method.

Requirements: Nails, cord or string, metre scale, hammer, pencil, notebook.

Procedure

Method:

i. Prepare a L-shaped structure of 1 × 1 metre size in the given area by using 3 nails and tying them with a cord or string.

ii. Measure 10 cm on one side of the arm L and the same on the other side of L, and prepare 10 x 10 sq. cm area using another set of nails and string. Note the number of species in this area of 10 x 10 sq. cm.

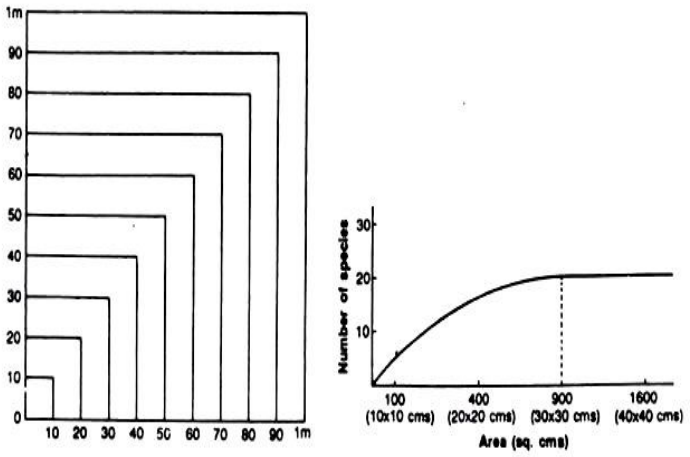


Fig. 67. A, Procedure for determining minimum required size of the quadrat; B, Species area-curve to determine the size of the quadrat.

iii. Increase this area to 20 × 20 sq. cm and note the additional species growing in this area.

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iv. Repeat the same procedure for 30 × 30 sq. cm, 40 × 40 sq. cm and so on till 1 × 1 sq. metre area is covered (Fig. 67) and note the number of additional species every time.

Record your data in the following table:

No.	Area	Total no. of species
1.	10 × 10 sq. cm	
2.	20 × 20 sq. cm	
3.	30 × 30 sq. cm	
4.	40 × 40 sq. cm	
:	:	
10.	100 × 100 sq. cm	

v. Prepare a graph using the data recorded in the above table. Size of the quadrats is plotted on X- axis and the number of species on Y-axis.

Observations:

The curve starts flattening or shows only a steady increase (Fig. 67 B) at one point in the graph.

Results:

The point of the graph, at which the curve starts flattening or shows only a steady or gradual increase, indicates the minimum size or minimum area of the quadrat suitable for study.

2.2 TO DETERMINE MINIMAL NUMBER OF QUADRATES BY SPECIES AREA CURVE METHOD IN FOREST AND GRASSLAND

Aim: To determine species area curve for sampling of population by quadrate method.

Requirements: quadrate of definite size, graph sheet, pencil, scale.

Principle:

The minimum size of the quadrate is equally determined by the species area curve method. The size of the quadrate is very important as too small or too large quadrate may not be representative of the community.

Procedure:

The procedure is to lay a quadrate of small area in the sampling plot and the occurrence of the number of the species is observed. The

plotting area is independent varieties (X-axis) and the occurrence of number of species, then plotting the area as the dependent variable (Y-axis)

The minimum quadrate size can be determined then the curve takes a horizontal shape indicating that the species number does not increase. The point where the curve flattens is joined with the X-axis. To find out the minimum area corresponding to the occurrence of maximum number of species. This method is very convenient for vegetation and analysis or plant analysis.

a. A quadrate of definite size is thrown randomly in the area provided for sampling of the species composition at different sites, all the different types of the species and their quantity. It determines the different species concept.

b. Calculate the total number of individuals of a species and total number of quadrate of occurrence of a species.

c. Calculate the total species types on each quadrate i.e. quadrate no and the new types of the species to the previous no. of quadrate.

d. Now plot the species area curve on a graph paper.

Result:

The minimum of the two quadrate are required for complete sampling of the given areas curve.

2.3 SAMPLING OF THE GRASSLAND VEGETATION BY QUADRAT METHOD FOR DETERMINING THE DENSITY, FREQUENCY AND BASAL COVER OF DIFFERENT SPECIES

Aim: To study communities by quadrat method and to determine density, frequency and basal cover

Requirements: Metre scale, string, four nails or quadrat, notebook.

(i) Frequency:

Frequency is the number of sampling units or quadrats in which a given species occurs.

Percentage frequency (%F) can be estimated by the following formula:

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$$\% \text{ frequency (F)} = \frac{\text{Number of quadrats in which the species occurred}}{\text{Total number of quadrats studied}} \times 100$$

(ii) Density:

Density is the number of individuals per unit area and can be calculated by the following formula:

$$\text{Density (D)} = \frac{\text{Total number of individuals}}{\text{Total number of quadrats studied}}$$

Fig. 68. A wooden quadrat of 50 x 50 cm.

(iii) Abundance:

Abundance is described as the number of individuals per quadrat of occurrence.

Abundance for each species can be calculated by the following formula:

$$\text{Abundance (A)} = \frac{\text{Total number of individuals}}{\text{Number of quadrats of occurrence}}$$

(iv) Basal area of a plant species is calculated by the following formula:

Basals area of a species = $p\ r^2$

Where p = 3.142, and r = radius of the stem

Procedure

Method:

Lay a quadrat (Fig. 68) in the field or specific area to be studied. Note carefully the plants occurring there. Write the names and number of individuals of plant species in the note-book, which are present in the limits of your quadrat. Lay at random at least 10 quadrats (Fig. 69) in the same way and record your data in the form of Table 4.1.

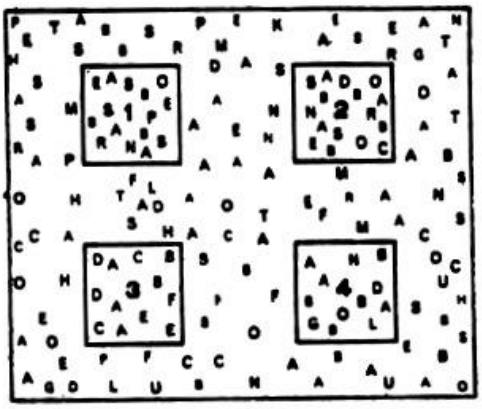

Fig. 69. Sketch of an artificial field showing four quadrats (1–4).

In Table 4.1, % frequency, density and abundance of Cyperus have been determined. Readings of the other six plants, occurred in the quadrats studied, are also filled in the table. Calculate the frequency, density and abundance of these six plants for practice. (For the practical class take your own readings. The readings in Table 4.1 are only to give an explanation of the matter).

Results:

Calculate the frequency, density and abundance of all the plant species with the help of the formulae given earlier and note the following results:

(i) In terms of % Frequency (F), the field is being dominated by…

(ii) In terms of Density (D), the field is being dominated by…

(iii) In terms of Abundance (A), the field is being dominated by…

Observations:

Table 4.1: Size of quadrat: 50cm × 50cm = 2500 cm^2

S. No.	Name of plant species	Number of individuals in quadrat number										Total number of quadrats of occurrence	Total number of quadrats studied	Total number of individuals	% Frequency (F)	Density (D)	Abundance (A)
		1	2	3	4	5	6	7	8	9	10						
1.	*Cyperus*	10	9	7	0	0	3	8	15	0	7	7	10	60	70%	6	8.57
2.	*Cassia*	0	0	2	0	3	0	5	0	6	10						
3.	*Cynodon*	50	0	7	41	6	0	0	8	0	5						
4.	*Eclipta*	0	0	4	0	3	0	0	1	0	2						
5.	A	0	0	0	0	2	0	0	1	3	0						
6.	B	5	10	1	0	0	0	3	1	0	2						
7.	C	3	5	0	0	2	1	8	0	2	0						

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2.4 TO DETERMINE DIVERSITY INDICES (SPECIES RICHNESS, CONCENTRATION OF DOMINANCE, SIMPSON, SHANNON-WIENER, EQUITABILITY AND B DIVERSITY) FOR PROTECTED AND UNPROTECTED GRASSLAND STANDS.

Aim: To determine diversity indices (richness, Simpson, Shannon-Wiener) for protected grazed and protected grassland.

Requirements: Khurpa, note book, pen.

Procedure:

Method and results:

(a) Species diversity: Species diversity is a statistical abstraction with two components. These two components are:

(i) Richness (or number of species), and

(ii) Evenness or equitability.

In any grassland, to be studied, if there are seventy species in a stand, then its richness is seventy. Pick out individual plants of different species with the help of khurpa, count the number of species in a stand of the area provided, and calculate the richness. On the other hand, if all the species in the grassland have equal number of individuals, then its evenness or equitability is high and if some species have only a few individuals then the evenness is low.

(b) Index of dominance:

The species which have strongest control over energy flow and environment in given habitat are called ecological dominant. According to Simpson (1949), the Index of dominance (C) is calculated by the formula

$C= \sum (ni/N)^2$

where$\sum$ (sigma) refers to summation, ni refers to the importance value of the species in terms of number of individuals or biomass or productivity of each species over a unit area, and N refers to the total of corresponding importance values of all the component species in the

same unit area and period. Count the Index of Dominance by the above-mentioned formula.

(c) Similarity index:

Similarity Index between two stands of vegetation can be worked out by the formula S = 2 C/(A+B), where S is the Similarity Index, C is the number of species common to both the stands, and A and B are number of species on stand A and stand B. For example, if there are 20 species on site A and 20 on site B and 14 species are common in both sites, the Similarity Index (S) will be;

S = 28/40 or S = 0.7.

(d) Dissimilarity index:

The Dissimilarity Index is counted by the formula D = 1 – S, where D is the dissimilarity index and S is the similarity index. For example, if there are 20 species on site A and 20 species on site B and 14 species are common in both sites the similarity index (S) comes to 0.7 as calculated above in case of similarity index. Therefore, dissimilarity index (D) can be counted, as

D= 1-S or D= 1-0.7 = 0.3.

(e) Diversity index:

Species diversity index (d) is calculated by the following formula given by Menhinick (1964):

$d=S/\sqrt{N}$

where d = diversity index, S = number of species, and N= number of individuals of that particular species.

Simpson's Diversity Indices:

The term "Simpson's diversity index" can actually refer to any one of 3 closely related indices.

Simpson's Index (D):

Simpson's index measures the probability that any two individuals drawn at random from an infinitely large community will belong to same species. There are two versions of the formula for calculating D.

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Either is Acceptable but is to be Consistent:

$$D = \sum (n/N)^2 \qquad D = \frac{\sum n(n-1)}{N(N-1)}$$

where, n = the total number of individuals of each species, N = the total number of organisms of all species.

The value of D ranges between 0 and 1.

With this index, 0 represents infinite diversity and 1, no diversity. That is, the bigger the value of D, the lower the diversity. This does not sound logical, so to get over this problem, D is often subtracted from 1 or the reciprocal of the index is taken.

Simpson's Index of Diversity 1-D:

This index represents the probability that two individuals randomly selected from a community will belong to different species. The value of this index also ranges between 0 and 1, but here, the greater the value, the greater the diversity.

Simpson's Reciprocal Index 1/D:

The value of this index starts with 1 as the lowest possible figure. This figure would represent a community containing only one species. The higher the value, the greater would be the diversity. The maximum value is the number of species in the sample. For example, if there are five species in the sample, then maximum value is 5.

The name Simpson's diversity index is often very loosely applied and all three related indices described above (Simpson's index, Simpson's index of diversity and Simpson's reciprocal index) have been quoted under term, depending on authors.

As an example, let us consider the following table:

Species	Number(n)	n(n – 1)
A	2	2
B	8	56
C	1	0
D	1	0
E	3	6
Total (N)	15	64

Putting the values into the formula for Simpson's index:

$$D = \frac{\sum n(n-1)}{N(N-1)} = \frac{64}{15 \times 14} = 0.3 \text{ (Simpson's index)}$$

Then, Simpson's index of diversity 1 – D = 0.7 and Simpson's reciprocal index 1/D = 3.3.

All these three values represent the same biodiversity. It is, therefore, important to ascertain which index has actually been used in any comparative studies of biodiversity. The disadvantage of Simpson's index is that it is heavily weighted toward the most abundant species, as are in all dominance indices.

The addition of rare species with one individual will fail to change the index. As a result, Simpson's index is of limited value in conservation biology if an area has many rare species with just one individual.

Shannon Index:

A widely used diversity index is Shannon index.

The Index is given by:

$$H_s = \sum_{i=1}^{s} p_i \ln p_i$$

Where, p_i is the proportion of individuals found in the i^{th} species and In denotes natural logarithm.

The following table gives an example:

	Species	Abundance	p_i	$p_i \ln p_i$
	A	50	0.5	– 0.347
	B	30	0.3	– 0.361
	C	10	0.1	– 0.230
	D	9	0.09	– 0.217
	E	1	0.01	– 0.046
Total	5	100	1.00	– 1.201

Putting the values into the formula for Shannon index, $H_s = 1.201$

Even the rare species with one individual (species E) contributes some value to the Shannon index, so if an area has many rare species, their contributions would accommodate. Shannon index has a minus sign in the calculation, so the index actually becomes 1.201, not-1.201. Values

of Shannon index for real communities are often found to fall between 1.5 and 3.5. The value obtained from a sample is in itself of no significance. The index becomes useful only while comparing two or more sites.

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2.5 TO ESTIMATE THE IVI OF THE SPECIES IN A WOODLAND USING POINT CENTRED QUARTER METHOD

Aim

To estimate the IVI of the species using Point centered quarter method

Introduction

Ecologists often need to estimate the number of individuals present in a population or community. If there are a few, large individuals, this can be done directly by counting each individual. If there are many individuals, the community is large, or the individuals are small, it may be impractical to count all of the individuals. In these cases it is easier to take samples of the community and then estimate density the population size based on the samples. For example, suppose that you wanted to know how many sugar maple trees are in the Adirondack Park. It would be impossible to count every tree in the 2.4 million hectare (6,000,000 acre) park. Instead, randomly-located samples can be taken. Suppose that 100 one-hectare samples were taken at random locations in the park and in each of these samples, the number of Maple trees was counted. The average number of maples per one-hectare sample was 500. From this we can estimate the number of maples in the entire park by multiplying 500 X 2,400,000. Plot sampling as described above may be difficult for sampling trees, particularly if a large area is to be sampled. It may be easiest to sample trees using a plotless technique. Plotless techniques are best used with stationary organisms because sometimes they involve measuring distances to organisms.

Principle

The point-quarter technique is perhaps the most popular of the plotless sampling techniques. Each sample is taken at a random location

in the area to be sampled. This is frequently done by choosing random points along a transect, but any randomization technique may be used.

Procedure

The area near each random point (sample point) is divided into four imaginary quadrants as indicated below. Within each quadrant, the nearest tree is included in the field sample.

There are four quadrants, so you will measure a total of four trees at each sample point. In the diagram below, point A represents a random point (sample point) and the letters b through h represent trees. The tees b, d, e and h would be included as the four nearest trees within each quadrant that are nearest to A.

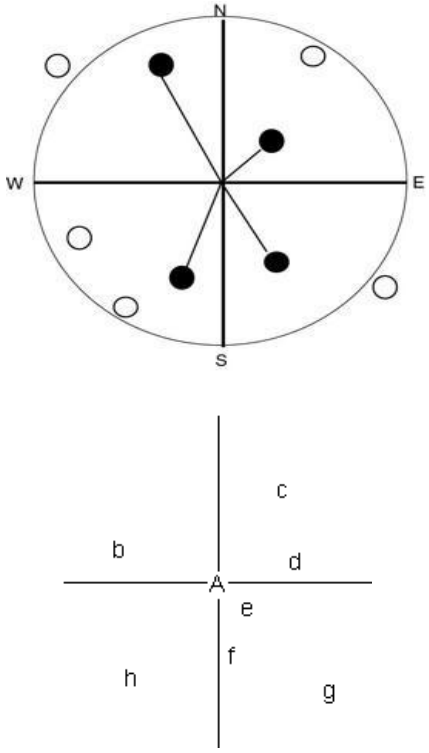

Diagram of point-centered quarter sampling. Sampled trees are indicated by solid (black) circles; trees not sampled are indicated by white circles. Within each of the four quadrants, the researcher locates the nearest tree to the sampling point in the center.

If you wish to measure tree density and/or basal area, you should also measure the distance from your sampling point to each tree, and the DBH of each tree. DBH is measured using a diameter tape wrapped around the tree at 1.3m above the ground. These measurements can then be used to

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calculate basal area by converting diameter to area and summing the measurements for all trees within a given area.

Tree Community analysis:

In order to enumerate the mushroom diversity, line transect method will be adopted for tree inventory. Each transect is subdivided into 20 X 20 m units to facilitate the tree inventory. All the trees ≥ 10 cm girth at breast height (GBH, 1.3 m) will measure and their girths record. The present study majority of the low gph trees also having mushroom diversity hence low statures of trees (gph, ≥ 10 cm) are accounted for measuring. Meter sticks will be used for measuring the line transect and to avoid the double counting, after complete the first transect the following transects will laid after 5 m intervals. Each tree (gph, ≥ 10 cm) will be tagged with the aid of sequentially numbered aluminum tag and trunk marked where measurements taken. If the trees having multiple stems each could be measured separately and basal area calculated and summed (Parthsarathy and Sethi, 1997).

Quantitative analysis:

The quantitative analysis such as density, frequency and abundance of tree species, will be determined as per Curtis and McIntosh (1950).

Density:

Density is number of individuals per unit area and can be calculated by the following formula

$$\text{Density (D)} = \frac{\text{Total number of individuals}}{\text{Total number of quadrats studied}}$$

Frequency (%):

Frequency is number of individual species present in all the studied quatrats and can be calculated by the following formula

$$\%\ \text{frequency (F)} = \frac{\text{Number of quadrats in which the species occurred}}{\text{Total number of quadrats studied}} \times 100$$

Abundance:

It is the study of the number of individuals of different species in the community per unit area and can be calculated by the following formula

$$\text{Abundance (A)} = \frac{\text{Total number of individuals}}{\text{Number of quadrats of occurrence}}$$

Importance Value Index:

This index is used to determine the overall importance of each species in the community structure. In calculating this index, the percentage values of the relative frequency, relative density and relative dominance are summed up together and this value is designated as the Importance Value Index or IVI of the species (Curtis, 1959).

Relative density:

Relative density is the study of numerical strength of a species in relation to the total number of individuals of all the species and can be calculated as

Relative density (RD) of a species is calculated by the following formula:

Relative frequency:

$$\text{RD} = \frac{\text{Total number of individuals of a particular species in all quadrats}}{\text{Total number of individuals of all the species in all quadrats}} \times 100$$

The degree of dispersion of individual species in an area in relation to the number of all the species occurred.

Relative density (RD) of a species is calculated by the following formula:

$$\text{RF} = \frac{\text{Number of quadrats in which a species occurs}}{\text{Total number of all the species in the quadrat}} \times 100$$

Relative dominance:

Dominance of a species is determined by the value of the basal cover. Relative dominance is the coverage value of a species with respect to the sum of coverage of the rest of the species in the area.

Relative dominance of a species is calculated by the following formula:

$$\text{Relative dominance} = \frac{\text{Total basal area of a particular Relative species in 5 quadrats}}{\text{Total basal area of all the species in 5 quadrats}} \times 100$$

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Basal area of a plant species is calculated by the following formula:

Basals area of a species = p r^2

Where p = 3.142, and r = radius of the stem

Calculate the IVI by adding these three values:

IVI = relative frequency + relative density + relative dominance.

2.6 TO DETERMINE THE GROSS AND NET PRODUCTIVITY OF PHYTOPLANKTON BY DARK AND LIGHT BOTTLE METHOD

Aim: To determine the gross and net productivity of phytoplankton by dark and light bottle method

Estimation of primary productivity using diel method (light and dark bottle method):

Principle:

Primary production of a water body is the rate of conversion of solar energy to chemical energy (organic compounds) by green plants. We usually distinguish between gross primary production and net primary production. Gross primary production is the rate of all photosynthetic processes regardless of the fate of the compounds which are produced during photosynthesis.

6H2O + 6 CO2 (g) + photon energy~→ C6H12O6 + 6 O2 (g)

Part of the photosynthate is diverted to growth (i.e., increase in biomass) and part is reconverted to CO2 and water in the process of respiration, which provides the energy to run the metabolic machinery of the cells. Net production is the total amount of photosynthesis which is diverted toward growth, and is thus always less than gross production. The relationship between gross production, net production and respiration is as follows:

Gross primary productivity (GPP) (mgC m-3 hr-1) = [12 x (LB – DB) x 1000]/ 32 x PQ x t Net primary productivity (NPP) (mgC m-3 hr-1) = [12 x (LB – DB) x 1000]/32 x PQ x t and **Respiration (mgC m-3 hr-1) = [12 x (LB – IB) x 1000]/ 32 x RQ x t**

Where, IB = Dissolved oxygen in the initial bottle in mg/L, DB = Dissolved oxygen in the dark bottle in mg/L, LB = Dissolved oxygen in the light bottle in mg/L, PQ = Photosynthetic quotient (1.2), RQ = Respiration quotient (1.0) and t = Time of incubation in hours.

In studying natural freshwater/brackish water/marine habitats, it is most useful to know all three components of the above equation. Typically only two components are measured directly, and the third is obtained by subtraction. The oxygen difference technique for determining primary production is based on this principle.

Reagents:

The reagents used for the quantification of primary productivity are essentially same as the ones used in the estimation of dissolved oxygen content described in section 2.2 as it is basically the titrimetric determination of D.O. in initial, light and dark bottles following various incubation periods.

Procedure:

To determine primary production at a particular depth in any water body, three sets of bottles were required: the initial bottles, the light bottles (i.e., those that admit light), and the dark bottles (i.e., bottles which are darkened with foil or tape so that they admit no light). A fresh sample was taken from the depth of interest with a water sampler and was added to each of the three types of bottles. Usually each bottle type is duplicated or triplicated. The amount of time the bottles spend in the direct sun was absolutely minimized while the bottles are being filled. Excessive exposure of the bottles to sunlight may damage the plankton and thus result in erroneous data. The light and dark bottles were filled in with the water without trapping any air bubble and were allowed to incubate for four hours. As soon as possible after these bottles are in place, the initial bottles are treated with Winkler reagents. The initial bottles would provide a measure of the oxygen content of the water at the beginning of the experiment. For the best results, the light and dark bottles were placed in a transparent tub or bucket containing a portion of

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the water in order to create a continuity of the reflection of light in and around the bottle so that the bottles didn't get over heated under sunlight.

The length of the incubation is determined by the productivity of the water body that is being studied. In very productive waters such as the one under study, short incubations produce enough oxygen in the light bottle to be detected by the Winkler technique. Short incubations usually span three to four hours. In less productive lakes longer incubations may be necessary, but incubations longer than 8 hours are highly undesirable because the artificial conditions inside the bottle resemble conditions in the lake less and less as time goes by. In some oligotrophic or unproductive waters, such as mountain lakes, it may not even be possible to use the oxygen difference method for detecting photosynthesis and respiration. In this case the Carbon-14 method must be used because of its greater sensitivity to low production rates.

The start of the incubation period was noted carefully. After the incubation had been in progress for a predetermined number of hours, the bottles were removed from the river and the time is noted once again. The bottles are immediately treated with Winkler reagents to stop all biological processes and fix the oxygen.

All of the bottles were titrated carefully for oxygen determination. Typically two different titrations were performed on each bottle. The dark bottles would almost always exhibit a decline in the amount of dissolved oxygen from the initial condition. Since photosynthesis is impossible in a dark bottle, only respiration would have occurred in the dark bottles. The decline of oxygen in the dark bottles is a quantitative measure of the amount of respiration which occurred over the incubation period. It should be noted, however, that this respiration includes that not only by plants but also animals and bacteria. Fortunately, the respiration of plants (algae) usually predominates in plankton systems and the respiration in the bottle is a pretty good approximation of the total respiration by plants.

In the light bottles, both respiration and photosynthesis had been occurring simultaneously. Since subtracting total photosynthesis from

respiration gives net photosynthesis, these light bottles indicated the amount of net primary production. Since the increase or decrease of oxygen in the light and dark bottles with reference to the initial oxygen conditions gives us a measure of respiration and net primary productivity, it is possible to calculate gross primary productivity using the equation given earlier. In the calculations it is customary to report respiration, net production, and gross production all in terms of milligrams of oxygen per liter per incubation period.

2.7 TO DETERMINE SOIL MOISTURE CONTENT, POROSITY, AND BULK DENSITY OF SOILS COLLECTED FOR VARYING DEPTH AT DIFFERENT LOCATIONS

Aim: Determine the natural content of the given soil sample.

1. Determination of Soil Moisture Content

Need and Scope of the Experiment

In almost all soil tests natural moisture content of the soil is to be determined. The knowledge of the natural moisture content is essential in all studies of soil mechanics. To sight a few, natural moisture content is used in determining the bearing capacity and settlement. The natural moisture content will give an idea of the state of soil in the field.

Definition

The natural water content also called the natural moisture content is the ratio of the weight of water to the weight of the solids in a given mass of soil. This ratio is usually expressed as percentage.

Apparatus Required

1. Non-corrodible air-tight container.
2. Electric oven, maintain the temperature between 1050 C to 1100 C.
3. Desiccator.
4. Balance of sufficient sensitivity.

Procedure

1. Clean the container with lid dry it and weigh it (W1).

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2. Take a specimen of the sample in the container and weigh with lid (W2).

3. Keep the container in the oven with lid removed. Dry the specimen to constant weight maintaining the temperature between 1050 C to 1100 C for a period varying with the type of soil but usually 16 to 24 hours.

4. Record the final constant weight (W3) of the container with dried soil sample. Peat and other organic soils are to be dried at lower temperature (say 600) possibly for a longer period.

Certain soils contain gypsum which on heating loses its water if crystallization. If it b is suspected that gypsum is present in the soil sample used for moisture content determination it shall be dried at not more than 800 C and possibly for a longer time.

OBSERVATIONS AND RECORDING

Data and observation sheet for water content determination

S.No.	Sample No.	1	2	3
1	Weight of container with lid W1 gm			
2	Weight of container with lid +wet soil W2 gm			
3	Weight of container with lid +dry soil W3 gm			
4	Water/Moisture content W = [(W2−W3)/(W3−W1)]□100			

RESULT

The natural moisture content of the soil sample is __

REMARKS

1. A container without lid can be used, when moist sample is weighed immediately after placing the container and oven dried sample is weighed immediately after cooling in desiccator.

2. As dry soil absorbs moisture from wet soil, dried samples should be removed before placing wet samples in the oven.

2. Determination of porosity:

Aim: To estimate the porosity of different soil samples:

Principle:

Porosity:

Pores between the soil particles are of great importance for the plant growth because they contain air and water. Porosity of the soil depends upon the texture and structure of the soil, compactness of the soil and also on the organic content of the soil. It increases with the increase in the percentage of organic matter in the soil. It decreases with the decrease in the size of the soil particles. Porosity also decreases with the depth of the soil in the earth.

Requirements:

Balance, oven, soil samples, measuring cylinders, water.

Procedure:

Method:

1. Take some amount of different soil samples (e.g. sand, clay), put them in the oven for some time and allow them to dry.
2. Weigh 250 gm. of each oven-dry soil samples and place each of them in separate measuring cylinders.
3. Add 250 ml of water in each measuring cylinder gradually through the wall and note the level of water in each cylinder.
4. Wait for about 15 minutes and again note the water level in each cylinder. There is a fall of water level in each cylinder. Now deduct the second water level from the first one. This will give us the porosity of soil sample.

Observations:

Table 4.10 :

Soil sample	First water level	Second water level	Porosity of soil	% porosity
1. Sand	A	E	A – E	$\frac{A - E \times 100}{250}$
2. Silt	B	F	B – F	$\frac{B - F \times 100}{250}$
3. Clay	C	G	C – G	$\frac{C - G \times 100}{250}$
4. Garden soil	D	H	D–H	$\frac{D - H \times 100}{250}$

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Result:

The percent porosity of sand, silt, clay and garden soil is…,…,…, and… respectively.

3. Determination of bulk density:

Dry weight of unit volume of soil inclusive of pore spaces IS called bulk density. It is expressed in terms of gm per ml or lbs per cubic foot. It is lesser than the particle density of the soil. Bulk density of soil may be calculated as: weight of soil/ volume of soil.

Aim: To estimate the bulk density of the grassland and woodland soil:

Requirements:

Soil samples, khurpa or any other digging instrument, oven, measuring cylinder, physical balance.

Method:

What is Bulk Density?

The dry weight of unit volume of the soil is called its bulk density. It is expressed as gram per cm^3. High bulk density is inversely proportional to the pore surface of the soil. The soils with bulk density ($gm./cm^3$) varying between 1.1 and 1.5 are medium to fine-textured while those between 1.5 and 1.7 are coarse-textured. Alkaline soils usually have a bulk density above 1.7. The bulk density is calculated by the formula:

Bulk density ($gm./cm^3$) = Weight of dry soil (gm.)/Volume of dry soil (cm^3)

Under mentioned is the procedure to find out the bulk density of the soil:

1. Take soil samples randomly from an area from a depth of about 15 cm.
2. Dry the soil in an oven for about 24 hours at a temperature of 105°C.
3. Transfer a part of the dried soil to a measuring cylinder and determine its volume.
4. Determine the weight of the above volume of soil on a balance.

Results:

Find the bulk density of the soil by the formula mentioned above. Also determine the type of the soil by the details mentioned above.

2.8 TO DETERMINE THE WATER HOLDING CAPACITY OF SOILS COLLECTED FROM DIFFERENT LOCATIONS

Aim: To determine water-holding capacity of given soil sample

Requirements:

Soil sample, oven, tin or brass box with perforated bottom, weighing box, weighing balance, filter paper, petri-dish.

Method:

1. Take the soil sample, crush it thoroughly and allow it to dry.
2. Take a tin or brass box with perforated bottom and weigh it.
3. Also take a filter paper and weigh it with weighing balance.
4. Put the weighed filter paper at the bottom of the tin or brass box and fill the box with soil.
5. Now place the soil-filled tin or brass box in a petri-dish containing water and wait for about 6 hours.
6. Again weigh the box containing wet soil.
7. Now put the box containing wet soil in an oven adjusted at 105°C and wait for about 24 hours.
8. Now remove this box and weigh it.
9. Now take four filter papers from the same filter paper packet used earlier, dip one of them in water and weigh it. In a similar fashion weigh other three wet filter papers and find out the average weight of water absorbed by one filter paper.

Observations:

(1) Weight of tin or brass box = 50 gm.

(2) Weight of dry filter paper = 0.12 gm.

(3) Weight of box + wet filter paper + wet soil = 142 gm.

(4) Weight of box + dry filter paper + dry soil = 122 gm.

(5) Weight of wet filter paper = 0.62 gm.

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Results:

Water-holding capacity is calculated as under:

(6) Weight of wet soil = (weight of box + wet filter paper + wet soil) – (weight of box + weight of wet filter paper)

= No. (3)-No. (1+5)

= 142 gm. – (50 gm. + 0.62 gm.)

= 142 gm.-50.62 gm.

=91.38 gm.

(7) Weight of soil dried in oven = (weight of box + dry filter paper + dry soil) – (weight of box + weight of dry filter paper)

= No. 4-No. (1 + 2)

= 122 gm – (50 gm + 0.12 gm)

= 122gm-50.12gm =71.88 gm

(8) Water present in the soil = weight of wet soil – weight of soil dried in oven

= No. 6 – No. 7 =91.38 gm – 71.88gm = 19.50 gm

(9) Water-holding capacity of the soil sample

= Amount of water present in the soil (8)/Weight of soil dried in oven (7) × 100

= 19.50/71.88 × 100%

= 27.12%

2.9 TO DETERMINE THE PERCENT ORGANIC CARBON AND ORGANIC MATTER IN THE SOILS OF CROPLAND, GRASSLAND AND FOREST LAND

Aim: To determine the percent organic carbon and organic matter in the soils.

Principle:

The organic matter present in the soil is digested with excess of potassium dichromate and sulphuric acid and the residual unutilized dichromate is then titrated with ferrous ammonium sulphate.

Procedure:

1. 10 gm of oven-dry soil is taken in 500 ml conical flask and to which 10 ml of 1 N $K_2Cr_2O_7$ solution (49.04 g of $K_2Cr_2O_7$ in 1 liter water) and 20 ml conc. H_2SO_4 is added. Then the mixture is shaken.
2. kept the flask for reaction (30 min approx.).
3. Then the mixture is diluted with dist. water up to 200 ml and then 10 ml phosphoric acid and 1 ml of diphenyl amine indicator (0.5 gms diphenyl amine in 100 ml H_2SO_4 and 20 ml water) is added.
4. The solution became dark blue, which is then titrated against 0.4 N ferrous ammonium sulphate [156.86 g $Fe(NH_4)_2 (SO_4)_2 6H_2O$ is dissolved in dist. water by adding 14 ml conc. H_2SO_4 and volume made up to 1 lit]. The end point of the titration is noted by change of colour of mixture from blue to brilliant green.
5. A blank set is run in the same manner without soil sample.

(Potassium dichromate solution content may be subjected to alteration depending on the carbon content of soil samples.)

Finally carbon content and organic matter are determined by the following formula:

Carbon (%) = 3.951/8 (1 – T/S)

Organic matter (%) = % C × 1.724

Where, g = weight of sample in gram

S = ml of ferrous solution with blank titration

T = ml of ferrous solution with sample titration.

Organic Carbon:

Soil organic carbon is a measureable component of soil organic matter. Organic matter makes up just 2–10% of most soil's mass and has an important role in the physical, chemical and biological function of agricultural soils. Organic matter contributes to nutrient retention and turnover, soil structure, moisture retention and availability, degradation of pollutants, carbon sequestration and soil resilience.

Determination of Soil Organic Carbon:

Take 1 g of soil in a 500 mL conical flask.

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- Add 10 mL of 1N $K_2Cr_2O_7$ solution and shake to mix it.
- Then add 20 mL Con. H_2SO_4 and swirl the flask 2 or 3 times.
- Allow the flask to stand for 30 minutes on an asbestos sheet for the reaction to complete.
- Pour 200 mL of water to the flask to dilute the suspension. Filter if it is expected that the end point of the titration is not to be clear.
- Add 10 mL of 85% H_3PO_4 and 1 mL of Diphenylamine indicator and back titrate the solution with 0.5 N Ferrous Ammonium Sulphate, till the colour flashes from violet through blue to bright green. H_3PO_4 gives sharper endpoint, by making the colour change, distinct through a flocculating effect.
- Note the volume of Ferrous Ammonium Sulphate.
- Carryout blank titration (without soil) in a similar manner.

i. Calculation:

% of Organic Carbon in Soil (R) is,

$$R = \frac{(V_1 - V_2) \times N \times 0.003 \times 100}{W} \times C$$

Where,

W - Weight of Sample

V_1 - Blank Titre value

V_2 - Titre value of the Sample

N - Normality of $K_2Cr_2O_7$ (Here it is 1N)

C - Correction Factor (1.334, 1.724)

2.10 TO ESTIMATE THE DISSOLVED OXYGEN CONTENT IN FRESH WATER BY AZIDE MODIFICATION OF WINKLER'S METHOD

Aim: To estimate dissolved oxygen (DO) content in the given water sample by Winkler's method.

Principle: Dissolved oxygen (DO) determination measures the amount of dissolved (or free) oxygen present in water or wastewater. Aerobic bacteria and aquatic life such as fish need dissolved oxygen to survive. If the amount of free or DO present in the wastewater process is too low,

the aerobic bacteria that normally treat the sewage will die. DO is determined by the titrimetric method developed by Winkler.

1. Dissolved molecular oxygen in water is not capable of reacting with KI, therefore an oxygen carrier such as manganese hydroxide is used. $Mn(OH)_2$ is produced by the action of KOH on $MnSO_4$.
2. $Mn(OH)_2$ so obtained reacts with dissolved molecular oxygen to form a brown precipitate of basic manganic oxide, $MnO(OH)_2$.
3. $MnO(OH)_2$ then reacts with concentrated sulphuric acid to liberate nascent oxygen.
4. Nascent oxygen results in oxidation of KI to I_2.
5. This liberated iodine is then titrated against standard sodium thiosulphate solution using starch as an indicator.
6. Thiosulphate reduces iodine to iodide ions and itself gets oxidized to tetrathionate ion. (Refer the reactions below.)

Dissolved oxygen should be measured as quickly and carefully as possible. Ideally, samples should be measured in the field immediately after collection.

Reagent List:

- 2ml Manganese sulfate
- 2ml alkali-iodide-azide
- 2ml concentrated sulfuric acid
- 2ml starch solution
- Sodium thiosulfate

These reagents are available in dissolved oxygen field kits, such as those made by the Hach Company. Please use caution when using these reagents, as they can be hazardous to one's health.

Procedure:

1. Carefully fill a 300-mL glass Biological Oxygen Demand (BOD) stoppered bottle brim-full with sample water.
2. Immediately add 2mL of manganese sulfate to the collection bottle by inserting the calibrated pipette just below the surface of the liquid. (If the reagent is added above the sample surface, you will introduce oxygen into the sample.) Squeeze the pipette slowly so no bubbles are introduced via the pipette.

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3. Add 2 mL of alkali-iodide-azide reagent in the same manner.
4. Stopper the bottle with care to be sure no air is introduced. Mix the sample by inverting several times. Check for air bubbles; discard the sample and start over if any are seen. If oxygen is present, a brownish-orange cloud of precipitate or floc will appear. When this floc has settle to the bottom, mix the sample by turning it upside down several times and let it settle again.
5. Add 2 mL of concentrated sulfuric acid via a pipette held just above the surface of the sample. Carefully stopper and invert several times to dissolve the floc. At this point, the sample is "fixed" and can be stored for up to 8 hours if kept in a cool, dark place. As an added precaution, squirt distilled water along the stopper, and cap the bottle with aluminum foil and a rubber band during the storage period.
6. In a glass flask, titrate 201 mL of the sample with sodium thiosulfate to a pale straw color. Titrate by slowly dropping titrant solution from a calibrated pipette into the flask and continually stirring or swirling the sample water.
7. Add 2 mL of starch solution so a blue color forms.
8. Continue slowly titrating until the sample turns clear. As this experiment reaches the endpoint, it will take only one drop of the titrant to eliminate the blue color. Be especially careful that each drop is fully mixed into the sample before adding the next. It is sometimes helpful to hold the flask up to a white sheet of paper to check for absence of the blue color.
9. The concentration of dissolved oxygen in the sample is equivalent to the number of milliliters of titrant used. Each mL of sodium thiosulfate added in steps 6 and 8 equals 1 mg/L dissolved oxygen.

Results Analysis:

The total number of milliliters of titrant used in steps 6-8 equals the total dissolved oxygen in the sample in mg/L. Oxygen saturation is temperature dependent - gas is more soluble in cold waters, hence cold

waters generally have higher dissolved oxygen concentrations. Dissolved oxygen also depends on salinity and elevation, or partial pressure.

2.11 TO ESTIMATE THE CARBON DIOXIDE EVOLUTION FROM DIFFERENT SOIL BY USING SODA LIME OR ALKALI ABSORPTION METHOD

Aim: To estimate the carbon dioxide from different soil by using soda lime or alkali absorption method.

Principle: The principle of this method is that CO_2 liberated by respiratory tissue is removed from its chamber along with CO_2 free gas stream and absorbed in baryta (Barium hydroxide solution) taken in Pettenkoffer's tube to form barium carbonate. This is then titrated by a standard acid (HCL) to know its CO_2 content.

Requirements:

Jars, respiratory substrate, Pettinkoffer's apparatus, barium hydroxide solution, wooden stand, pressure regulator (suction pump), grease, soda lime, oxalic acid, phenolphpthalein, barium carbonate, caustic potash, burette, beakers, measuring cylinder, balance with weighing box.

Procedure

1. Fill the jars with soda lime and place about 100 gm. respiratory materials in U-tube chamber.
2. Now fill the long and narrow Pettinkoffer's tubes with solution of barium hydroxide of known concentration (N/10). Place the Pettinkoffer's tubes in such a way on a wooden stand that they are oriented obliquely.
3. Connect the tubes with a suction pump or pressure regulator.
4. Make the entire apparatus air-tight by applying grease.
5. Now allow the pressure regulator to function. Due to this the air current rushes into the jars filled with soda lime which absorbs carbon dioxide of the air. The air now passes through the plant material placed in the U-shaped respiratory chamber (Fig. 53).

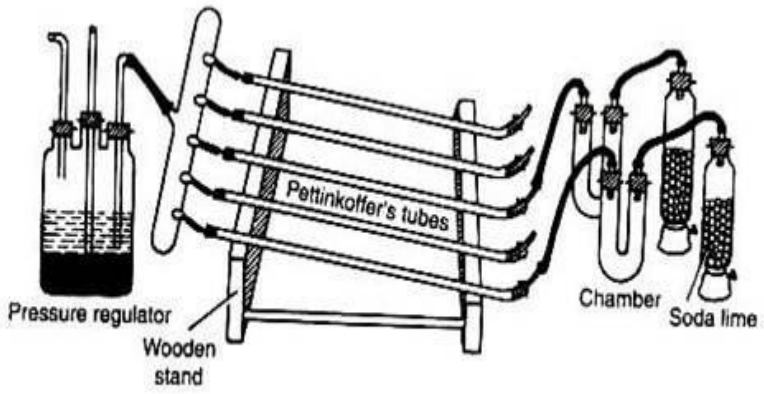

Fig. 53. To measure the rate of respiration by volumetric method using Pettinkoffer's tubes.

6. From the U-shaped chambers, the air is now bubbled through the Pettinkoffer's tubes filled with barium hydroxide solution.

7. For regulating a slow movement of air and bubbles through the soda lime, respiratory substrate and barium hydroxide solution, the pressure regulator is allowed to work. The air first passes through one of the Pettinkoeffer's tube and then it is allowed to flow through the second tube.

8. Now remove the first tube and titrate its contents. Due to the presence of precipitated barium carbonate ($BaCO_3$) the contents become turbid.

9. Measure about 25 ml. of contents of Pettinkoffer's tube, i.e., barium carbonate and titrate it against N/10 solution of oxalic acid.

10. Phenolphthalein drops are used as indicator in the beaker containing barium carbonate and note the end point.

Observations and results:

Observations and results may be noted in the form of following table:

S.No.	Barium carbonate volume (cc)	Oxalic acid volume used (cc)		Volume used (cc)
		Initial reading (cc)	Final reading (cc)	
1.	25	0	10	10
2.	25	11	20	9
3.	25	21	32	11
4.	25	33	42	9
5.	25	43	54	11
Average :				10 cc

$N_1V_1 = N_2V_2$, where

(a) N_1 = Known normality of oxalic acid.

It is calculated as follows:

Molecular weight of oxalic acid = 126

Equivalent weight 126/2 = 63

Therefore, N = 63

N/10= 6.3 gm. of oxalic acid dissolved in 1000 cc of water

So, N, = 6.3.

(b) V_1 = Known volume of oxalic acid used = 10 cc

(c) N_2 = Normality of barium hydroxide solution, which is to be determined.

(d) V_2 = Volume of barium carbonate =25 cc

In this way $N_1V_1 = N_2V_2$ may be calculated as follows:

$6.3 \times 10 = N_2 \times 25$

Therefore, $N_2 = 6.3 \times 10 / 25 = 2.52$

Conclusion:

The amount of CO_2 produced by 100 gm. of given plant material (respiratory substrate) is 2.5 mg/litre in one hour. In the same way, rate of respiration of different respiratory substrates or different plant parts can be determined.

3. Algal Technology and Mushroom Technology

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3.1 ISOLATION AND CULTIVATION OF FRESH AND MARINE WATER ALGAE

Aim: To isolate and cultivate fresh water algae

Principle:

Microalgae belong among the fastest-growing photosynthetic organisms since their cell doubling time can be as little as a few hours. Biomass production by algae (oxygenic photosynthetic organisms) is based on the simple scheme shown below, which determines all the necessary requirements of this biological process:

$CO_2 + H_2O$ + nutrients + light energy $\rightarrow$ biomass + O_2

In biotechnology, generally, the production of biomass requires well-defined conditions. The necessary cultivation requirements for the growth of algal mass cultures are light, a suitable temperature and pH, and a sufficient supply of carbon and nutrients in the growth medium. Since algal mass cultures grow in dense suspensions, some kind of turbulent mixing is necessary to expose cells to light and to allow for an efficient mass transfer.

Procedure

Field collection:

Water samples with visible algal populations were collected from ponds, lakes, and rivers. Sampling of large bodies of fresh water occurred at multiple sites along the waterfront. Collections were made for the top and bottom of the water at each location, with the goal of determining the dominant algal species in each area. All field samples were collected in 50 mL tubes and maintained at refrigerated condition while transferring to laboratory.

Isolation of fresh water Algae:

In order to isolate single algal species from the field water samples, standard plating methods were used to separate algal

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populations. Multiple media recipes were utilized to isolate the colonies. The field samples were first diluted to aid in the isolation process. Sterilized plastic petri dishes (100 × 15 mm) containing approximately 40 mL of agarized medium were used to plate these diluted samples. One milliliter of the diluted sample was transferred to a media plate and spread evenly across the surface. Inoculated plates were placed in a temperature-controlled greenhouse (20-25°C, approximately 27 μE/m/s) where the algae were allowed to grow for about 14 days. Grown algae cultures were streaked using sterile technique onto additional sets of nutrient media plates and placed back in the greenhouse for isolation. This streaking method was repeated until isolation into axenic unialgal cultures was achieved. The number of colonies that were transferred from each dilution plate onto other nutrient media plates depended on the amount of contamination and the identification of the colonies present based on the colony morphology and the microscopic cellular morphology of each isolate. Following the isolation of individual microalgae colonies, each strain was initially labeled based on the sampling location and special media requirements. Isolated algae were maintained as stock cultures and were stored on a cool, low light shelf. These stock cultures were maintained by re-plating each onto new nutrient media at least once a month, or more frequently depending on the nature of each isolated strain.

Culture medium/nutrients:

Concentrations of cells in phytoplankton cultures are generally higher than those found in nature. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies in the seawater. Macronutrients include nitrate, phosphate (in an approximate ratio of 6:1), and silicate.

Table. A generalized set of conditions for culturing micro-algae (modified from Anonymous, 1991).

Parameters	Range	Optima
Temperature (°C)	**16-27**	**18-24**
Salinity ($g.l^{-1}$)	**12-40**	**20-24**
Light intensity (lux)	**1,000-10,000 (depends on volume and**	**2,500-5,000**

	density)	
Photoperiod (light: dark, hours)		**16:8 (minimum) 24:0 (maximum)**
pH	**7-9**	**8.2-8.7**

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Silicate is specifically used for the growth of diatoms which utilize this compound for production of an external shell. Micronutrients consist of various trace metals and the vitamins thiamin (B_1), cyanocobalamin (B_{12}) and sometimes biotin. Two enrichment media that have been used extensively and are suitable for the growth of most algae are the Walne medium (Table) and the Guillard's $F/_2$ medium (Table). Various specific recipes for algal culture media are described by Vonshak (1986). Commercially available nutrient solutions may reduce preparation labour. The complexity and cost of the above culture media often excludes their use for large-scale culture operations. Alternative enrichment media that are suitable for mass production of micro-algae in large-scale extensive systems contain only the most essential nutrients and are composed of agriculture-grade rather than laboratory-grade fertilizers

Table. Composition and preparation of Walne medium (modified from Laing, 1991).

Constituents	**Quantities**
Solution A (at 1 ml per liter of culture)	
Ferric chloride ($FeCl_3$)	0.8 g[(a)]
Manganous chloride ($MnCl_2$, $4H_2O$)	0.4 g
Boric acid (H_3BO_3)	33.6 g
EDTA[(b)], di-sodium salt	45.0 g
Sodium di-hydrogen orthophosphate (NaH_2PO_4, $2H_2O$)	20.0 g
Sodium nitrate ($NaNO_3$)	100.0 g
Solution B	1.0 ml
Make up to 1 litre with fresh water[(c)]	Heat to dissolve

Solution B	
Zinc chloride ($ZnCl_2$)	2.1 g
Cobaltous chloride ($CoCl_2$,6 H_2O)	2.0 g
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}$, $4H_2O$)	0.9 g
Cupric sulphate ($CuSO_4$, $5H_2O$)	2.0 g
Concentrated HCl	10.0 ml
Make up to 100 ml fresh water[(c)]	Heat to dissolve
Solution C (at 0.1 ml per liter of culture)	
Vitamin B_1	0.2 g
Solution E	25.0 ml
Make up to 200 ml with fresh water[(c)]	
Solution D (for culture of diatoms-used in addition to solutions A and C, at 2 ml per liter of culture)	
Sodium metasilicate (Na_2SiO_3, $5H_2O$)	40.0 g
Make up to 1 litre with fresh water[(c)]	Shake to dissolve
Solution E	
Vitamin B_{12}	0.1 g
Make up to 250 ml with fresh water[(c)]	
Solution F (for culture of *Chroomonas salina* - used in addition to solutions A and C, at 1 ml per liter of culture)	
Sodium nitrate ($NaNO_3$)	200.0 g
Make up to 1 litre with fresh water[(c)]	

(a) Use 2.0 g for culture of *Chaetoceros calcitrans* in filtered sea water;

(b) Ethylene diamine tetra acetic acid;

(c) Use distilled water if possible.

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Table. Composition and preparation of Guillard's $F/_2$ medium (modified from Smith *et al.*, 1993a).

Nutrients	**Final concentration ($mg.l^{-1}$ seawater)[a]**	**Stock solution preparations**
$NaNO_3$	75	Nitrate/Phosphate Solution Working Stock: add 75 g $NaNO_3$ + 5 g NaH_2PO_4 to 1 liter distilled water (DW)
$NaH_2PO_4.H_2O$	5	
$Na_2SiO_3.9H_2O$	30	Silicate Solution Working Stock: add 30 g Na_2SiO_3 to 1 liter DW
$Na_2C_{10}H_{14}O_8N_2.H_2O$ (Na_2EDTA)	4.36	Trace Metal/EDTA Solution Primary stocks: make 5 separate
$CoCl_2.6H_2O$	0.01	1-liter stocks of ($g.l^{-1}$ DW) 10.0 g $CoCl_2$, 9.8 g
$CuSO_4.5H_2O$	0.01	$CuSO_4$, 180 g $MnCl_2$, 6.3 g Na_2MoO_4, 22.0 g $ZnSO_4$
$FeCl_3.6H_2O$	3.15	
$MnCl_2.4H_2O$	0.18	Working stock: add 1 ml of each primary stock solution + 4.35 g $Na_2C_{10}H_{14}O_8N_2$ + 3.15 g $FeCl_3$ to 1 liter DW
$Na_2MoO_4.2H_2O$	0.006	
$ZnSO_4.7H_2O$	0.022	
Thiamin HCl	0.1	Vitamin Solution Primary stock: add 20 g thiamin HCl + 0.1 g biotin + 0.1 g B_{12} to 1 liter DW
Biotin	0.0005	
B_{12}	0.0005	Working stock: add 5 ml primary stock to 1 liter DW

Light

As with all plants, micro-algae photosynthesize, *i.e.* they assimilate inorganic carbon for conversion into organic matter. Light is the source of energy which drives this reaction and in this regard intensity, spectral quality and photoperiod need to be considered. Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture (*e.g.* 1,000 lux is suitable for erlenmeyer flasks, 5,000-10,000 is required for larger volumes). Light may be natural or supplied by fluorescent tubes. Too high light intensity (*e.g.* direct sun light, small container close to artificial light) may result in photo-inhibition. Also, overheating due to both natural and artificial illumination should be avoided. Fluorescent tubes emitting either in the blue or the red light spectrum should be preferred as these are the most active portions of the light spectrum for photosynthesis. The duration of artificial illumination should be minimum 18 h of light per day, although cultivated phytoplankton develop normally under constant illumination.

pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2-8.7. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished by aerating the culture (see below). In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth.

Aeration/mixing

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (*e.g.* in outdoor cultures) and to improve gas exchange between the culture medium and the air. The latter is of primary importance as the air contains the carbon source for photosynthesis in the form of carbon dioxide. For very dense cultures, the CO_2 originating from the air (containing 0.03% CO_2) bubbled through

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the culture is limiting the algal growth and pure carbon dioxide may be supplemented to the air supply (*e.g.* at a rate of 1% of the volume of air). CO_2 addition furthermore buffers the water against pH changes as a result of the CO_2/HCO_3^- balance. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, erlenmeyers), aerating (bags, tanks), or using paddle wheels and jetpumps (ponds). However, it should be noted that not all algal species can tolerate vigorous mixing.

Temperature

The optimal temperature for phytoplankton cultures is generally between 20 and 24°C, although this may vary with the composition of the culture medium, the species and strain cultured. Most commonly cultured species of micro-algae tolerate temperatures between 16 and 27°C. Temperatures lower than 16°C will slow down growth, whereas those higher than 35°C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with refrigerated air - conditioning units.

Salinity

Marine phytoplankton are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting sea water with tap water. Salinities of 20-24 $g.l^{-1}$ have been found to be optimal.

Nutrient media

A number of different nutrient media recipes were utilized to isolate the maximum number of colonies from each field sample. These nutrient recipes were modified in order to optimize the isolation and growth dynamics of each colony. Common media recipes were used in the first stages of the isolation procedure, including a modified Guillard's f/2 medium. Additional nutrient media were prepared in the laboratory: Jaworski's medium, Von Stosch's medium modified following Guiry and Cunningham and Bold's Basal medium with vitamins. The general nutrient medium f/2 was purchased as two parts premixed products from

the Aquatic Ecosystems (Apopka, Florida), and supplemented with nitrogen and phosphorus by adding Miracle-Gro plant food at a final concentration of 0.1 g/L. Guillard's f/2 were prepared using distilled water for the purpose of selecting organisms that are capable of growing in minimal nutrients, the composition contains vital nutrient salts (to top off the nutrients of sea water) required for the algal growth.

Cyanobacteria in field samples were also isolated by using nutrient media recipes specifically optimized for the growth of cyanobacteria species. These media recipes were similar in their absence of nitrogen in any form in the actual recipe. The BG-11_0 medium was prepared from the modified BG-11 medium recipe by removing all forms of nitrogen from the mixture in order to support only heterocystous cyanobacteria. BB-LU_0 containing no nitrogen was derived from the Bold's Basal medium with vitamins. The final two nutrient media recipes ($CyLU_0$-1SM and $CyLU_0$-10SM) were composed of the dehydrated seawater (sea minerals) concentrate at 1 or 10 g/L concentrations, respectively, in addition to 0.4 g/L of $K_2HPO_4 \cdot 3H_2O$ and 15 g/L of agar. All nutrient media recipes were sterilized in an autoclave prior to inoculation, except for the seawater concentrate, which was filter sterilized and added after autoclaving the other portions of the media. Distilled water was used unless otherwise specified in the recipe.

Cultivation and harvesting:

Each algal culture sample was monitored every day for cellular growth rates by measuring optical density at 680 nm. A spectrophotometer was used after calibrating it with a sample of the F/2 and Miracle Gro mixture media as blank. The cultures were continuously aerated using air pumps with air stones, and the specified media was added to each culture at the end of every week. Constant mixing of the algal culture in the tank was provided by the aeration. The temperature of the mass culture of algae in the tank remained between 21°C and 32°C. Large volume harvesting of the microalgae was achieved by the flocculation of algae after adding 2 mg chitosan per liter of algal culture and subsequent gravimetric settlement.

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To isolation and cultivation of marine water algae

Aim: To isolate and cultivate of marine water algae

Introduction:

The majority of algae that are intentionally cultivated fall into the category of microalgae (also referred to as phytoplankton, microphytes, or planktonic algae). Macroalgae, commonly known as seaweed, also have many commercial and industrial uses, but due to their size and the specific requirements of the environment in which they need to grow, they do not lend themselves as readily to cultivation (this may change, however, with the advent of newer seaweed cultivators, which are basically algae scrubbers using upflowing air bubbles in small containers). Commercial and industrial algae cultivation has numerous uses, including production of food ingredients such as omega-3 fatty acids or natural food colorants and dyes, food, fertilizer, bioplastics, chemical feedstock (raw material), pharmaceuticals, and algal fuel, and can also be used as a means of pollution control. Global production of farmed aquatic plants, overwhelmingly dominated by seaweeds, grew in output volume from 13.5 million tonnes in 1995 to just over 30 million tonnes in 2016.

Water, carbon dioxide, minerals and light are all important factors in cultivation, and different algae have different requirements. The basic reaction for algae growth in water is carbon dioxide + light energy + water = glucose + oxygen + water. This is called autotrophic growth. It is also possible to grow certain types of algae without light; these types of algae consume sugars (such as glucose). This is known as heterotrophic growth.

Principle:

Microalgae belong among the fastest-growing photosynthetic organisms since their cell doubling time can be as little as a few hours. Biomass production by algae (oxygenic photosynthetic organisms) is based on the simple scheme shown below, which determines all the necessary requirements of this biological process:

$$CO_2 + H_2O + \text{nutrients} + \text{light energy} \longrightarrow \text{biomass} + O_2$$

In biotechnology, generally, the production of biomass requires well-defined conditions. The necessary cultivation requirements for the growth of algal mass cultures are light, a suitable temperature and pH, and a sufficient supply of carbon and nutrients in the growth medium. Since algal mass cultures grow in dense suspensions, some kind of turbulent mixing is necessary to expose cells to light and to allow for an efficient mass transfer.

Procedure

Washing method or centrifugation: Repeated washing or centrifuging the water samples results in the isolation of larger organisms.

By exploiting the phototactic movement: By this method, the phytoflagellates will move to one direction and with a micropipette can be isolated.

By agar plating method: For preparing the agar medium, 1.5 % agar is added to 1 L of suitable medium or even natural seawater, and this agar solution is sterilized in an autoclave for 15 min under 150 lbs pressure and 120º C temperature. Then this medium is poured in sterilized Petri dishes and left for 24 h. In case of culture tubes, the medium is poured in 1/3 part in tubes and properly plugged with cotton before autoclaving.

Micromanipulation: The algal cell is to be isolated in drop of enrichment sample. While observing, the cell is sucked up into micropipette. The cell is transferred to a drop of sterile medium on agar plate. This process is repeated to "wash" the cell. The more times a cell is washed, the less likely is bacterial contamination. However, the risk of cell damage increases with the number of times a cell is handled. The optimum number of washes will depend on the type of algae. Then transfer the cell to dilute medium in a tissue culture plate, Petri dish, or culture tube. Culture vessel is placed under low light at appropriate constant temperature. Growth is checked under the microscope, or we have to wait until macroscopic growth can be detected (3–4 weeks after transfer). A colonial unialgal culture results from this method. Serial dilution: Tubes have to be labeled as 10^{-1} –10^{-10} to indicate dilution factor. Aseptically 1 ml of enrichment sample is to be added to the test

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tube (10^{-1}) and mixed gently. 1 ml of this dilution is taken and added to the next tube (10^{-2}) and then mixed gently. This procedure is repeated for the remaining tubes (10^{-3} – 10^{-10}). Test tubes are incubated under controlled temperature and light conditions: The cultures are examined microscopically after 2–4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture may grow in one of the higher-dilution tubes, e.g., 10^{-6} – 10^{-10}. If the tubes contain two or three different species, then micromanipulation can be used to obtain unialgal cultures.

Light:

As with all plants, microalgae photosynthesize, i.e., they assimilate inorganic carbon for conversion into organic matter. Light is the source of energy which drives this reaction, and in this regard intensity, spectral quality, and photoperiod need to be considered. Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the algal culture: at higher depths and cell concentrations, the light intensity must be increased to penetrate through the culture (e.g., 1,000 lux is suitable for Erlenmeyer flasks; 5,000–10,000 is required for larger volumes). Light may be natural or supplied by fluorescent tubes. Too high light intensity (e.g., direct sunlight, small container close to artificial light) may result in photoinhibition. Also, overheating due to both natural and artificial illumination should be avoided. The duration of artificial illumination should be minimum 18 h of light per day, although cultivated phytoplankton develops normally under constant illumination. In controlled rooms, white daylight fluorescent lamps may be used. Outdoor cultures rely on sunlight for illumination.

Temperature:

Temperature usually affects an organism's metabolic rate. Low temperatures are usually maintained in controlled rooms (18–23º C). Transfer of algal starters or inoculate previously cultured in controlled rooms when scaled up for mass production should be done early morning to avoid stress brought about by sudden temperature increase. The

optimal temperature for phytoplankton cultures is generally between 20º and 24º C, although this may vary with the composition of the culture medium, the species, and the strain cultured. Most commonly cultured species of microalgae tolerate temperatures between 16º and 27º C. Temperatures lower than 16º C will slow down growth, whereas those higher than 35º C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with refrigerated air-conditioning units.

pH:

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished by aerating the culture. In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth. **Aeration/Mixing:**

Mixing is necessary to prevent sedimentation of the algae, to ensure that all the cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (e.g., in outdoor cultures), and to improve gas exchange between the culture medium and the air. The latter is of primary importance as the air contains the carbon source for photosynthesis in the form of carbon dioxide. For very dense cultures, the CO_2 originating from the air (containing 0.03 % CO_2) bubbled through the culture is limiting the algal growth, and pure carbon dioxide may be supplemented to the air supply (e.g., at a rate of 1 % of the volume of air). CO2 addition furthermore buffers the water against pH changes as a result of the CO_2/HCO_3 balance. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, Erlenmeyer), aerating (bags, tanks), or using paddle wheels and jet pumps (ponds). However, it should be noted that not all algal species can tolerate vigorous mixing.

Carbon Dioxide:

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Providing the algae with extra carbon, in the form of the gas carbon dioxide (CO_2), would facilitate much faster growth. CO_2 is supplied from compressed gas cylinders, and only very little is needed (about half of one percent) in the air supplied to the culture. The CO_2 has to be passed through a flowmeter to ensure that the amount used will keep the pH of the culture between 7.8 and 8.0. The pH can be checked with indicator papers, which change color with a change in pH, or with a pH meter. Both the air and the CO2 should be filtered through an in-line filter unit of 0.3–0.5 μm before entering the culture, as this helps to prevent other, possibly contaminating, organisms from getting into the cultures.

Salinity:

Marine phytoplanktons are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting seawater with tap water. Salinities of 20–24 $g.l^{-1}$ have been found to be optimal.

Nutrient Medium:

In laboratory cultures, however, natural waters themselves are unsatisfactory for sustained algal growth mainly because some essential nutrients are usually present only in trace amounts. The concentration of these elements largely depends on dynamic equilibrium which is disturbed as soon as water is collected. Miquel (1890–93) observed that the waters of lakes, ponds, and the sea could not support in the laboratory continued and luxuriant growth of algae. Natural waters have to be enriched by the addition of some mineral salts that compounded in the famous solutions A and B. This marked the beginning of the use of enriched culture media where specific conditions are imposed to encourage growth of particular organisms.

Culture Media:

The following are some of the culture media found suitable to most planktonic algae:

TMRL medium (Tung Kang Marine Res. Lab.)

Potassium nitrate	-	10 g/100 ml of DW
Sodium orthophosphate	-	1 g/100 ml of DW
Ferric chloride	-	0.3 g/100 ml of DW
Sodium silicate	-	0.1 g/100 ml of DW

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The chemicals are kept separately in 100 ml reagent bottle. 1 ml each to 1 l of sterilized seawater is added. This medium can be used for the mass culture of diatom.

Schreiber's medium

Potassium nitrate	-	0.1 g
Sodium orthophosphate	-	0.02 g
Soil extract	-	50 ml

Filtered and sterilized seawater l

Soil extract is prepared by boiling 1 kg of garden soil in 1 l of fresh water for 1 h. After 24 h, clear water is decanted and kept in a bottle. 50 ml of this soil extract can be added to each liter of sterilized seawater. This can be used as a medium while isolating the nanoplankton.

F/2 medium:

$NaNO_3$ (75.0 g/L dH2O)	-	1.0 ml
$NaH_2PO_4 \cdot H_2O$ (5.0 g/L dH_2O)	-	1.0 ml
$Na2SiO_3 \cdot 9H_2O$ (30.0 g/L dH_2O)	-	1.0 ml
F/2 trace metal solution	-	1.0 ml
F/2 vitamin solution	-	0.5 ml
Filtered seawater to	-	1.0 L

After all additions, the medium will be autoclaved.

F/2 trace metal solution

$FeCl_3 \cdot 6H_2O$	-	3.15 g
$Na_2EDTA \cdot 2H_2O$	-	4.36 g
$CuSO_4 \cdot 5H_2O$ (9.8 g/L dH_2O)	-	1.0 ml
$Na2MoO_4 \cdot 2H_2O$ (6.3 g/L dH_2O)	-	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (22.0 g/L dH_2O)	-	1.0 ml
$CoCl_2 \cdot 6H_2O$ (10.0 g/L dH_2O)	-	1.0 ml
$MnCl_2 \cdot 4H_2O$ (180.0 g/L dH_2O)	-	1.0 ml
Distilled water to	-	1.0 L

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F/2 vitamin solution

Vitamin B12 (1.0 g/L dH_2O)	-	1.0 ml
Biotin (0.1 g/L dH_2O)	-	10.0 ml
Thiamine HCl	-	200.0 mg
Distilled water to	-	1.0 L

Filter sterilizes into plastic vials, and stored in the refrigerator.

Note: F/2 medium contains extensive silica precipitate and should be used only when growing diatoms. For other algal groups, use F/2-Si medium.

Conway's or Walne's Medium

1. Nutrient solution A per liter of DW

$FeCl3.6H_2O$	-	1.3 g
$MnCl_2.4H_2O$	-	0.36 g
H_3BO_3	-	33.6 g
EDTA (disodium salt)	-	45.0 g
$NaH_2PO_4.2H_2O$	-	20.0 g
$NaNO_3$	-	100.0 g
TMS stock	-	1.0 ml

2. Trace metal solution B (TMS) per 100 ml DW

$ZnCl_2$	-	2.1 g
$CoCl_2.6H_2O$	-	2.0 g
$(NH_4)6Mo7O24.4H_2O$	-	0.9 g
$CuSO_4.5H_2O$	-	2.0 g

This solution is normally cloudy. Acidify with a few drops of conc. HCl to give a clear solution.

3. Vitamin solution C per 100 ml

Cyanocobalamin	-	10.0 mg
Thiamine 10.0 mg		
Biotin	-	200.0 μg
(micrograms)		
Medium per liter		
Nutrient solution (A)	-	1.0 ml
Trace metal solution (B)	-	0.5 ml

Vitamin solution (C)	-	0.1 ml
Sterilized seawater	-	1.0 l

Besides the above mentioned laboratory prepared chemical which serves as nutrients, commercial fertilizers can be used for the mass culture of diatoms and nano plankters, in open tanks for economic purpose. The media used for the open culture are:

Urea 46	-	10 mg/l
Super phosphate	-	10 mg/l
Ammonium sulfate	-	100 mg/l

3.2 CULTIVATION OF SPIRULINA AND AZOLA

Aim: To cultivate the *Spirulina* and *Azola*

Introduction:

Microalgae cultures have found its application for production of practical and potential metabolic products such as food supplements, lipids, enzymes, biomass, polymers, toxins, pigments, tertiary waste water treatment and "green energy" products. These products were achieved by cultivating the microalgae on diverse mineral media. Large open outdoor pond cultivation is one of the oldest industrial systems for algal cultivation for the production of single cell protein, health food, and β-carotene. Closed systems have been described for the production of biomass and some specialty chemicals under axenic conditions. Improvements in performance of photo bioreactor (PBR) systems have been obtained by optimizing illuminating techniques, efficient gas and liquidex change units, and controlled nutrient supply. The growth of microalgae can be limited by low levels of inorganic nutrients in poorly managed cultures in hatchery tanks or grow-out ponds. The biochemical composition of these microalgae can in turn to be influenced by these altered nutrient concentrations. When nitrogen is limiting, photo synthetically derived energy which is normally. channeled into producing more cells and more protein, is in part diverted into making storage products such as carbohydrate and lipid, most of which lack nitrogen.

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Spirulina is multicellular and filamentous blue green algae that has gained considerable popularity in the health and food industry. In addition it is also used as a protein and vitamin supplement to aquaculture diets. It grows in water, can be harvested and processed easily and has very high content of micro and macro-nutrients. This traditional food, known as 'dihe' was rediscovered in 'Chad' by a European scientific mission and is now widely cultured throughout the world. In many countries of Africa, it is still used as human food as a major source of protein and is collected from natural water, dried and eaten. It has gained considerable popularity in the human health and food industry. It has been used as a complementary dietary ingredient for fish, shrimp and poultry feeds. China is using this microalga as a partial substitute of imported forage to promote the growth, immunity and viability of shrimp. There has also been comprehensive research on the use of *Spirulina* as aquaculture feed additives in Japan.

Floating aquatic macrophytes are defined as plants that float on the water surface, usually with submerged roots. Floating species are generally not dependent on soil or water depth. *Azolla* is heterosporous free-floating freshwater fern that live symbiotically with *Anabaena azollae*, nitrogen-fixing blue-green algae. *Azolla* has a higher crude protein content (ranging from 19 to 30%) than most green forage crops and aquatic macrophytes. It has also attracted the attention of livestock, poultry and fish farmers. *Azolla* doubles its biomass in 3-10 days, depending on conditions, and easily reaches a standing crop of 8-10 tons/ha fresh weight in Asian rice fields; 37.8 tons/ha fresh weight (2.78 tons/ha dry weight) has been reported for *A. pinnata* in India. Like all other plants, *Azolla* needs all the micro and macro-nutrients for its normal growth and vegetative multiplication. All elements are essential; phosphorus is often the most limiting element for its growth. For normal growth, 0.06 mg/l/day are required. This level can be achieved in field conditions by the split application of super phosphate at 10-15 kg/ha.

20 mg/l is the optimum concentration. Macronutrients such as P, K, Ca and Mg and micronutrients such as Fe, Mo and Co have been shown to be essential for the growth and nitrogen fixation of *Azolla*. The present study, *S. platensis, C. vulgaris* and *A. pinnata* cultured by common culture techniques. These were subjected to analyse the nutrients profile such as crude protein, carbohydrate, lipid, profile of essential amino acids and minerals.

Materials and Methods Culture of *Spirulina platensis*:

Classification of *Spirulina platensis*

Phylum	:	Cyanobacteria
Class	:	Cyanobacteria
Sub class	:	Oscillatoria, Phycideae
Order	:	Oscillatoriales
Family	:	Spirulinaceae
Genus	:	*Spirulina*
Species	:	*S. platensis*

Collection and Cultivation of *Spirulina platensis*: The pure *S. platensis* culture were collected from *Spirulina* production research and training centre, Kadachanendal, Madurai, Tamil Nadu, India.

Preparation of inoculum: The microalgae, *S. platensis* was inoculated in *Spirulina* medium (Schlosser, 1994) (Table 3.2.1) (100 ml mother culture + 900 ml basal medium) and the cultures were incubated for 15 days at 24±1ºC in a thermo-statically controlled room and illuminated with cool inflorescence lamps (Phillips 40 W, cool daylight 6500 K) at an intensity of 2000 lux in a 12:12 h light dark regime.

Culture in plastic troughs: Culture troughs were cleaned well with bleach and were rinsed until bleach smell had totally gone off after the troughs were sun dried for 8 h. Later, the plastic troughs were filled with tap water up to 25 L and mixed well with the pure nutrient media (N-8) (Vonshak, 1986) (Table 3.2.1). 1 L of mother culture of *S. platensis* was inoculated in the plastic troughs. The plastic troughs were vigorously aerated to provide required quantity of oxygen and to keep cells and media in suspension. The required concentration of algae

developed after 30 days of inoculation. The plastic troughs were kept in open under 100% outdoor light exposure. A constant temperature of 25-30ºC was maintained throughout the growth period.

Counting of algal cells

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Sampling was performed once in five days using 10 ml capacity vials. *S. platensis* cells in each vial were preserved by adding 2-3 drops of formalin. One ml of sample was carefully filled in Neubauer Hemocytometer groove (Bauer, 1990) and covered with glass slide. The cells were enumerated under compound microscope. Hand tally counter was used for reliable counting. Algal cells were calculated by the following mathematical expression.

(Cells /ml-1) = Total number of cells counted/10×4×10-6].

Spirulina platensis mother culture medium

Spirulina platensis cultured in plastic troughs

Filtered *Spirulina platensis*

Dried *Spirulina platensis* powder

Component	*Stock Solution	Quantity used	Concentration in final medium
Solution 1	500 ml		
$NaHCO_3$		13.61g	$1.62x10^{-1}$
Na_2CO_3		4.03g	$3.80x10^{-2}$
K_2HPO_4		0.50g	$2.87x10^{-3}$
Solution 2	500 ml		
$NaNO_3$		2.5g	$2.94x10^{-2}$
K_2SO_4		1.0g	$5.74x10^{-3}$
NaCl		1.0g	$1.71x10^{-2}$
$MgSO_4.7H_2O$		0.2g	$8.11x10^{-4}$
$CaCl_2.2H_2O$		0.04g	$2.72x10^{-4}$
$FeSO_4.7H_2O$		0.01g	$3.60x10^{-5}$
$Na_2EDTA.2H_2O$		0.08g	$2.15x10^{-4}$
Trace metal solution (1ml)	(gl^{-1}		
$Na_2EDTA.2H_2O$		0.8g	$2.15x10^{-6}$
$FeSO_4.7H_2O$		0.7g	$2.52x10^{-6}$
$ZnSO_4.7H_2O$		1ml	$3.48x10^{-9}$
$MnSO_4.7H_2O$		1ml	$8.97x10^{-9}$
H_3BO_3		1ml	$1.62x10^{-7}$
$Co\ (NO_3)_2.6H_2O$		1ml	$3.44x10^{-9}$
$Na_2MoO_4.\ 2H_2O$		1ml	$4.13x10^{-9}$
$CuSO_4.\ 5H_2O$		1ml	$3.69x10^{-9}$
Vitamin solution (1ml)	(gl^{-1}	5mg	
Cyanocobalamine (vitamin B_{12})			

Table: *Spirulina* culture medium (Schlosser, 1994)

Source: Andersen (2005).

* Stocks were procured from *Spirulina* production, research and training centre

Kadachanendal, Madurai, Tamil Nadu, India

Culture of Azolla pinnata

Classification *Azolla pinnata*

Kingdom : Plantae

Sub kingdom : Tracheobionta

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Division	:	Pteridophyta (Ferns)
Class	:	Filicopsia
Order	:	Hydropteridales
Family	:	Azollaceae (Azolla family)
Genus	:	Azolla Lam. (Mosquito fern)
Species	:	*A. pinnata* (Feathered mosquito fern)

Materials: Wet clay soil, cow dung, urea, *Azolla* culture and plastic tubs.

Collection of pure mother culture *Azolla pinnata*: The pure cultures of *A. pinnata* were collected from *Azolla* cultivation and Research Centre, Tamil Nadu Agriculture University Coimbatore, Tamilnadu.

Culture of *A. pinnata* in troughs

A trough of 24 L was taken to which a sediment layer of 3 cm clay was made. 20L of water was poured into the trough. 0.5% of urea was dissolved in the water along with 1 kg cow dung extract. This composition was allowed to stand for 2 days. The trough was kept outdoor in direct sunlight. To this medium, *A. pinnata* cultures were added. The *A. pinnata* growth was monitored daily. The levels of medium were topped with tap water to compensate the evaporation losses on alternative days. After 30 days *A. pinnata* biomass was harvested and weighted and dried at 60ºC in a hot air oven till concordant weights were recorded.

***Azolla pinnata* culture**

Harvested *Azolla pinnata*

Azolla pinnata powder

3.3 IMMOBILIZATION OF ALGAE AND USE OF ALGAE AS BIOCARRIER

Aim: To study the immobilization of algae and the use of algae as bio carrier.

Principle

An immobilized cell is defined as a living cell that, by natural or artificial means, is prevented from moving independently from its original location to all parts of an aqueous phase of a system. The concealed concept is that immobilized microalgae in matrices may assist the required biotechnological applications from the mass culture of the microalgae, either a specific metabolite or removal of pollutants. This concept evolved from the basic nature of its components, the microalgae and the immobilizing matrix.

Introduction:

The use of microalgae in biotechnology has been increased in recent years, these organisms being implicated in food, cosmetic,

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aquaculture and pharmaceutical industries, but small size of single cells implies a problem in the application of biotechnological processes to those organisms. In order to solve those problems, cell immobilization techniques have been prospered. Most of the general immobilization techniques for microorganisms can be easily modified and applied to microalgae, adding a design factor that these are photosynthetic microorganisms that require light. Six different immobilization types have been defined: covalent coupling, affinity immobilization, adsorption, confinement in liquid– liquid emulsion, capture behind semi-permeable membrane, and entrapment in polymers. These types of immobilation can be grouped as ‘‘passive” (using the natural tendency of microorganisms to attach to surfaces – natural or synthetic – and grow on them) and ‘‘active” (flocculant agents, chemical attachment, and gel encapsulation).

Algae, immobilized in calcium alginate, provide a standardized amount of photosynthetic material, enabling semi quantitative experiments to be undertaken. The rate of carbon dioxide uptake by the immobilized cells is used to measure the rate of photosynthesis this can be done simply by observing the colour change of hydrogen carbonate indicator, either by eye or using a colorimeter. The effect of varying the intensity or wavelength of the light may be studied, as can the effect of temperature or cell density. Different species of algae or cyanobacteria may also be tested.

Equipment and materials

Equipment

10mL plastic syringe (without a needle), Small beakers or disposable plastic cups, 2, 100mL measuring cylinder, Tea strainer, Glass stirring rod or small plastic stirrer, 5–7mL bijou bottles, with caps, ~8, Bench lamp, Ruler and colorimeter

Materials

- 3% sodium alginate solution, 3mL
- 2% calcium chloride solution, 100mL
- Suspension of algae, e.g., *Scenedesmus quadricauda*, 50mL (which concentrates to 3mL)

Hydrogen carbonate indicator solution, ~45mL

- ✓ Hydrogencarbonate indicator Makes 1 litre of 10x stock solution
- ✓ Cresol red, 0.1g
- ✓ Thymol blue, 0.2g
- ✓ Sodium hydrogencarbonate (sodium bicarbonate, NaHCO3), 0.85g Ethanol 20mL
- ✓ Freshly-boiled distilled water ~1L
- ✓ Dissolve 0.1g of cresol red and 0.20g of thymol blue in 20mL of ethanol.
- ✓ Dissolve 0.85g of sodium hydrogencarbonate in ~200mL of freshly-boiled (and therefore CO2-free) distilled water.
- ✓ Add the ethanolic solution of cresol red and thymol blue and dilute to 1L with freshly-boiled distilled water.

For use, dilute this stock solution with nine volumes of freshly-boiled distilled water and adjust the pH to ~7.4. Ideally, this solution should be fully aerated before use so that it is a bright red colour.

Note: Hydrogen carbonate indicator is very sensitive to changes in pH, and it is therefore important that all glassware, etc is rinsed with a little of the indicator before use.

To prepare a set of standard solutions

If a colorimeter is not available, the colour change of the indicator can be semi-quantified by comparing it to a series of coloured buffered solutions. Solutions ranging from pH7.6–9.2 can be made using boric acid-borax buffer.

- 10mL vials with lids, 9
- Boric acid, 12.4g
- Sodium tetraborate decahydrate (Borax, Na2B4O7.10H2O),
- 19.5g 9mL stock hydrogencarbonate indicator solution
- ✓ Dissolve the boric acid in a litre of distilled or deionised water.
- ✓ Dissolve the borax in another litre of distilled or deionised water.
- ✓ To 25mL of the boric acid solution, add the volume of borax indicated in the table below and make up to 100mL with distilled or deionised water.

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- ✓ Place 9mL of each of the prepared solutions into each of a series of vials.
- ✓ Immediately before the lesson, add 1mL of the stock (that is, concentrated) hydrogencarbonate indicator solution to each vial. Students may compare their results with the colours in the tubes.

To culture the algae:

- 2 litre PET lemonade bottle
- Aquarium air pump
- Aquarium airline tubing and airstone or glass sparger
- Cotton wool or foam, ideally non-absorbent, for closing the top of the bottle
- Low-temperature lighting, e.g., 18W energy-saving bulbs (equivalent to 100W), 2
- Algal enrichment medium, 1L

- ✓ Add 1.5 g of the enrichment medium to 1 litre of distilled water in a 2 litre lemonade bottle and shake to dissolve.
- ✓ Some residual powder will settle out, this is normal and will be utilized by the algae as the soluble nutrients are depleted.
- ✓ Inoculate the bottle with the algae.
- ✓ Insert an airline to aerate the culture with an air pump. This will provide extra dissolved carbon dioxide and keep the algae circulating.
- ✓ Stopper the bottle with a loose cotton wool or foam bung. Continuously illuminate the culture with a bright light while it is growing. Small fluorescent strip lights or 18W low-energy lamps give good results. The best results are obtained when extraneous light in the laboratory is minimized.

Procedure:

- ✓ Prepare a concentrated suspension of algae. There are two ways to do this: Either Leave the 50mL of dark green algal suspension to settle out (ideally, overnight) then carefully pour off the supernatant to leave approximately 3mL of concentrate. Or Centrifuge 50mL of the suspension at low speed for 5 minutes.

Pour off the clear supernatant to leave approximately 3mL of concentrate.

- ✓ Pour ~3mL of the algal suspension into a small beaker and add an equal volume of sodium alginate solution. Stir gently until the algae are evenly distributed.
- ✓ Draw the algae/alginate suspension into a syringe.
- ✓ Place a beaker of calcium chloride solution under the syringe and allow the algae/alginate mixture to drip slowly from the syringe tip into the liquid below. Swirl the calcium chloride solution gently as this happens.
- ✓ Leave the beads of immobilised algae to harden in the calcium chloride solution for 5–10 minutes. The alginate molecules will be cross-linked by the calcium ions, trapping the cells in a matrix of calcium alginate.
- ✓ Separate the beads from the calcium chloride solution using the tea strainer and gently wash the beads with cold tap water. Give the beads a final rinse with distilled water. If they are illuminated and not allowed to dry out, the beads may be kept for several weeks. Alternatively, the beads may be kept in distilled water in a fridge for several months. Beads removed from the fridge should be allowed to warm up for about 30 minutes before use.
- ✓ Take several small bottles and rinse them with a small volume of hydrogencarbonate indicator solution.
- ✓ Add equal numbers of algal beads to each bottle, then add a standard, measured, volume of hydrogencarbonate indicator solution. Replace the lid of each bottle. Approximately 12–15 beads will be required in each tube.
- ✓ Place the containers at different light intensities by standing them at various distances from a bright light. Leave them for 1–2 hours until the indicator in some of the containers changes colour.
- ✓ Determine the relative carbon dioxide concentration in each tube with algal beads. There are two ways to do this: Either Match the colours of the hydrogencarbonate indicator in the tubes with algal

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beads with a range of standard solutions; Or Use a colorimeter to measure the absorbance of the solutions at 550nm (that is, using a green filter). Note: It is important to measure absorbance (not transmission) in these experiments since there is a linear response between absorbance and pH of the indicator over the range studied.

- ✓ Plot a graph to show the absorbance at 550nm, or the pH of the hydrogen carbonate indicator against the relative light intensity, that is, 1 /(distance2).

Uses of algae as biocarrier:

(a) Accumulation and removal of waste products in aqueous systems,

(b) Biosynthesis and biotransformation of different natural products such as polysaccharides, enzymes etc.

(c) Production of ammonia,

(d) Production of photosynthetic oxygen in combined bacteria-algae systems,

(e) Production of hydrogen.

It is well known that several algal species develop impressive capabilities of accumulating certain compounds, for instance heavy metals but also nitrogen (in the form of ammonia) and phosphorus (as orthophosphate), from the environment.

It can be shown that species of Scenedesmus, immobilized in carrageenan. are able to remove within a few hours high percentages of phosphate and ammonia from typical urban secondary effluents at a similar rate to free living cells. Similar results were obtained on the removal of heavy metals in industrial effluents, where the immobilizing matrix seems to protect the algae to a certain degree against toxic effects of the metal ions.

Most of the studies on immobilized algae deal with their use for biosynthesis and transformation of valuable biological compounds such as enzymes, polysaccharides, NADPH, amino acids and hydrocarbons. Glycolic acid production is a characteristic of all plants that fix CO_2 via the Calvin cycle. Studies on free-living algal cells have demonstrated that

the excretion of this compound occurs widely among photosynthetic organisms.

In *Chlorella* cells immobilized in Calcium alginate gel, glycolate production could be maintained over a period of six months. Other research groups have demonstarted the photoproduction of NADP by Nostoc muscorum, immobilized in polyurethane foam, the continuous production of amino acids over 10 days by different mutants of cyanobacteria, encapsulated in alginate, the long term release of sulfated polysaccharides from polyurethane-entrapped Porphyridium cruentum or the production of hydrocarbons by Botryococcus braunii. Dunaliella sp. is a marine microalga well-known for its potential to produce glycerol and β-carotene.

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Reported results suggest significant release of glycerol by this alga depending on parameters such as temperature, light intensity and salinity. Studies on *Dunaliella tertiolecta*, immobilized in Ca-alginate beads, showed to produce significant amounts of glycerol, even in hypersaline media (up to 4M NaCl) over a period of several months. In addition to the metabolites mentioned above, the production of other compounds such as pigments, vitamins, growth stimulants and antibiotics are projected as potential products from immobilized algae.

Another application of immobilized algae is the production of ammonia. Its production by N_2-fixing cyanobacteria, immobilized in alginate matrix, was described by using either inactivation of glutamine synthetase by L-methionine-D, L-sulfoximine deficient strains, showing a yield of 40% of fixed nitrogen excreted as ammonia.

Production of oxygen for 25 days was reported for *Chlorella vulgaris* and *Scenedesmus obliquus* after immobilization in urethane prepolymer, and a period of over six months for Chlorella emersonii entrapped in alginate gel. Oxygen production was also described for the immobilized red alga *Porphyridium cruentum*.

3.4 PREPARATION OF SEAWEED LIQUID BIOFERTILIZERS

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Aim: To Prepare the seaweed liquid biofertilizers

Introduction

A biofertilizer (also bio-fertilizer) is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances.

PRINCIPLE

Biofertilizers can be expected to reduce the use of synthetic fertilizers and pesticides. The microorganisms in biofertilizers restore the soil's natural nutrient cycle and build soil organic matter. Through the use of biofertilizers, healthy plants can be grown, while enhancing the sustainability and the health of the soil. Since they play several roles, a preferred scientific term for such beneficial bacteria is "plant-growth promoting rhizobacteria" (PGPR). Therefore, they are extremely advantageous in enriching soil fertility and fulfilling plant nutrient requirements by supplying the organic nutrients through microorganism and their byproducts. Hence, biofertilizers do not contain any chemicals which are harmful to the living soil.

Materials and Methods:

Seaweeds were collected and washed with sea water to remove unwanted impurities and other debris.

They were shade dried and brought to the laboratory and they were thoroughly washed with tap water for 3 to 4 times to remove all the epiphytes, sand particles and other fauna.

After drying, it was cut into small pieces and kept in hot air oven for one day at 60^{o} C. Then, it was made into coarse powder with help of mixer grinder.

The powdered seaweeds were made into liquid extracts as per the method of Seaweed Liquid Fertilizer (SLF)

Plant material Plants used in this work were two lettuce commercial varieties (*Lactuca sativa*), “Maravilhas de Inverno” (green lettuce) and “Maravilhas das Quatro Estações” (purple lettuce).

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Seaweed extracts two seaweeds, *Ascophyllum nodosum,* and *Sargassum muticum* were used as a source for biofertilizer. 1) Aqueous crude extract (ACE), obtained from fresh seaweeds triturated with distilled water and filtered, to remove the larger particles 2) Aqueous extract processed (AEP), obtained through chemical hydrolysis with ethanol and distilled water.

Obtaining the ACE:

The ACE is obtained hiyan by reducing the algal material to small particles suspended in distilled water, where all the constituents of the algae are present. Initially, the algae were cut using food scissors, in order to reduce its size. The algae were placed in a vessel and distilled water was added in the proportion of 1 600 mL of distilled water per kilogram of seaweed. The algae together with the distilled water were then placed until a “juice” was obtained, repeating the process until all algae were liquefied. The obtained juice was crushed again using a 750-W power wand and later filtered by a filter set consisting of a metal mesh strainer and two cloth strainers coupled to remove the particles larger dimensions. The obtained filtrate solution is then the ACE of the algae (pH 6, 3); this was stored at a temperature between 2°C and 4°C in properly labeled plastic bottles and sealed with parafilm.

Obtaining the AEP:

The initial protocol consisted of a sequential extraction, where the same sample is used from the beginning to the end of the process and first passes through a phase where it is extracted with hexane, another where it is extracted with methanol and finally an extraction with distilled water at 100°C. As this protocol entails high costs due to the required volumes of hexane and methanol, it has been determined that these solvents would be replaced with ethanol since, theoretically, the residue

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obtained in this solvent extraction has a similar content and, using a rotary evaporator with vacuum pump, it is possible to reuse the ethanol. At each extraction, a volume of 2 500 mL of aqueous extract was obtained, this process being repeated whenever more quantity was needed. Twenty-five grams of dry seaweed were added to 500 mL of ethanol (1:20 ratio) in a goblet with a magnet, stirring for 20 min at room temperature. After this time, the sample is allowed to stand for 1 min and filtration is carried out. The filtration is carried out in a vacuum, with the aid of funnel silica filters with G 3 porosity coupled to a Kitasato. Thus, under vacuum, the contents of the goblet are poured into the filter, where the solvent is recovered and stored into a hermetically sealed bottle, and the initial sample is retained in the silica filter. The recovered solvent is transferred into the Kitasato and into a properly labeled vial and closed with filter paper in order to avoid solvent losses. The dried sample retained in the silica filter is again placed in the goblet with the magnet is subjected to further extraction with ethanol, and this process is repeated until the ethanol solution is translucent. It should be noted that initially, the sample has a rather intense greenish coloration but as the process is repeated it becomes translucent because the solvent removes a large part of the polar and nonpolar compounds in the sample, to which the chlorophylls are associated and therefore the greenish color will "disappear". After extraction with ethanol, the aqueous extraction step was followed, in which distilled water was added to the sample in a ratio of 1:100. For this purpose, a graduated glass beaker is used to measure 2 500 mL of distilled water that is poured into an Erlenmeyer flask. The Erlenmeyer is placed on a heating plate until the distilled water reaches 100°C. At 100°C, the sample was added to the water and left for 2 h, with gentle stirring, for extraction to take place. After that time, the solution is allowed to cool and vacuum filtration using a silica filter with G 2 porosity coupled to a Kitasato (in the same manner as for ethanol extraction). The part of the sample that was trapped in the silica filter was discarded and the solution obtained (pH 6, 5) is recovered and stored at 4° C into a hermetically sealed bottle.

3.5 SURVEY ON MUSHROOMS

Aim: To survey the mushroom species in the field.

Introduction:

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Mushrooms are the fruiting bodies of basidiomycetous fungi and are specifically called basidiomata (sing, basioma). They are formed only during certain periods of the year when conditions are congenial, and are bright coloured. Mushrooms may be edible or non-edible, poisonous or non-poisonous. Mushrooms are cosmopolitan heterotrophic organisms that are quite specific in their nutritional and ecological requirements. As such, they have been generally divided into humicolous, lignicolous, coprophilous, fungicolous, parasitic or saprophytic or may show some mycorrhizal associations with both broad-leaved forest trees and gymnospermous taxa. They constitute the most relished food commodities amongst the number of non-conventional foodstuffs primarily because of their unique flavor and texture. Wild edible mushrooms have been collected and consumed by people since thousands of years. Archaeological evidences reveal edible species associated with people living 13000 years ago.

Materials and Methods:

Sampling of macrofungi

A group of 6 people, three at each side of the central line walked along transects the length of 20m each end and all macro fungi within the transect belt were recorded. We have used meter sticks to describe an area of 2m on both sides and walked forward the length of transects observed the type and number of individual of the species. To avoid the double counting, after complete the first transect the following transects were laid 5m intervals. Each macro fungi species within each transect was collected in separate specimen bags in order to avoid spore contamination among the different specimens, and these were photographed in colored and tagged. Morphological features such as size, color, shape, and texture of the sporocarps were recorded as these features might change with drying. Identification of specimen was based

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on macroscopic and microscopic features. The macroscopic features used were: the cap size, shape, color, surface texture and surface moisture, gill color, attachment, spacing, lamellules, the stem size, shape, surface texture and surface moisture, the presence or absence of partial and universal veils, flesh color and texture. Using standard protocols, diversity indices such as Simpson and Shannon-Weiner diversity indices were used. The samples are preserved in Formal-acetic Alcohol solution (FAA): consist of 13 formalin, 5ml of glacial acetic acid and 200 ml of ethyl alcohol (50%).

3.6 ISOLATION AND PURE CULTURE OF EDIBLE MUSHROOMS

Aim: To isolate and culture edible mushrooms

Isolation techniques for getting pure cultures and their maintenance: There are two methods to have a mushroom culture - the Spore Culture and Tissue Culture technique.

1. Spore Culture

a) Spore Print:

- In order to get a spore print or collection of spores, the cap from a healthy, disease free mushroom is removed , surface cleaned with a swab of cotton dipped in alcohol and placed on a clean sterilized white paper or on clean glass plate or on surface of the clean glass slides. The surface nearby should be thoroughly sterilized. To prevent air flow, place a glass jar or clean glass or cup over the cap surface. Spores will fall on the white paper or slide surface within 24-48 hours exactly like radial symmetry of the gills. The spore print on the paper can be preserved for a longer time by cutting and folding it into two halves.

b) Spore transfer and germination:

- In order to get a pure culture , the scalpel is sterilized by keeping it on a burning flame for 8-10 seconds till it becomes hot red, cool it by dipping in a sterilized medium, scrap some spores from the spore print taken on a paper or glass slide and transfer them by gently streaking on the agar medium aseptically. Minimum, three

agar dishes should be inoculated for each spore print and the culture developed after its incubation at appropriate temperature is known as multispore culture.

2. Tissue Culture

- A small bit from the pileal region is cut with the help of a sterilized blade or scalpel, washed several times in sterilized distilled water and dried in a clean tissue paper before inoculating aseptically on a Petri plate or tube containing suitable culture medium. The inoculated Petri plates are incubated at 25 ± I C for 6-12 days and observed at different intervals for the mycelial growth. All Petri plates / glass tubes showing contaminations should be discarded and only the ones with pure growth should be retained for further use after ascertaining the purity and true to type nature of the culture.

  Sub-culturing:

  The pure culture of edible mushroom, once established either through spore culture or tissue culture technique, is maintained properly in cool atmosphere or a refrigerator. Sub-culturing is done from time to time by aseptically transferring a small piece of growing pure culture along with the culture medium on the test tube slants containing same or other suitable medium. The pure culture of a mushroom can be used for preparing master cultures for large scale spawn production on commercial scale.

3.7 SPAWN PREPARATION FOR THE GROWTH OF MUSHROOMS

Aim: To prepare spawn essential for the growth of mushroom

Principle: In the spawn-production process, mycelium from a mushroom culture is placed onto steam-sterilized grain, and in time the mycelium completely grows through the grain. This grain/mycelium mixture is called spawn, and spawn is used to "seed" mushroom compost.

Materials required: Wheat or Sorghum grains, polythene sheet, 2% calcium sulphate (gypsum) and 0.5% calcium carbonate (chalk), milk bottles/ polypropylene bags,

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Procedure

Production of Spawn:

The spawn (seed of mushroom) is a pure culture of the mycelia grown on a special medium. The medium is prepared by the grains of wheat, rye, sorghum or bajra along with some ingredients.

The preparation of spawn mainly consists of three steps:

a. Preparation of substrate,

b. Inoculation of substrate, and

c. Incubation of inoculated substrate for spawn production.

Preparation of Substrate:

- Take 900 gms of grains (wheat or sorghum) in 600-900 ml of water in a container and boil for 15-20 minutes, After boiling, decant the excess water and allow the grains to surface drying by spreading on polythene sheet in shade for a few hours.
- The grains are then mixed with chemicals like 2% calcium sulphate (gypsum) and 0.5% calcium carbonate (chalk) on dry weight basis and adjust the pH of the grain at 7-7.8. About 300-350 gms grains were then filled in milk bottles/ polypropylene bags.
- Place a ring of tin (3.5 cm height and 3 cm diametre) towards the inner side of the open-end of polypropylene bag, tighten it with rubber band and then push the margin of the bag towards the inner side and thus a mouth is prepared.
- Plug the mouth of the bottle and/or polypropylene bag with non-absorbent cotton. Then cover the mouth with brown paper and tighten it with rubber band. Sterilize the substrate by autoclave at 15lb pressure for 30 minutes for 2 consecutive days. Kept the sterilized substrate in open air to cool down near to room temperature, thus making the substrate ready for inoculation.

Inoculation of Substrate:

The substrate is then inoculated with the mycelial culture (developed earlier, either in Potato Dextrose Agar i.e., PDA or Yeast

Potato Dextrose Agar i.e., YPDA or Malt extract Agar and Rice bran decoction medium).

Incubation:

Incubate the inoculated container at 20-25° C in dark for 3 weeks. Shake the container after a few days, when the mycelial growth becomes visible on the grain.

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Storage of Spawn:

Store the spawn at 0-4° C in a refrigerator for a maximum period of 6 months, if it is not needed immediately.

Precaution to be observed:

- Avoid over cooking of sorghum grains, as over cooking lead to splitting of grains.
- Use only recommended dose of CaCo3 for mixing with the cooked grains.

3.8 MASS CULTIVATION OF EDIBLE MUSHROOMS

Aim: To cultivate edible mushrooms in a mass level.

Principle:

Mushroom growing techniques require the correct combination of humidity, temperature, substrate (growth medium) and inoculum (spawn or starter culture). Wild harvests, outdoor log inoculation and indoor trays all provide these elements.

Procedure

Methods:

On Paddy Straw:

Paddy straw mushroom - *Volvariella volvacea* (Bull. ex. Fr.) Sing, cultivation comprises of the following stages preparation of spawn; preparation of mushroom bed; spawning and watering of mushroom bed; mushroom crop production; and harvesting of mushrooms.

i. Preparation of Spawn:

Mushroom spawn can be simply defined as a medium impregnated with mushroom mycelium and serves as the ‘seed’ or ‘inoculum’ for mushroom cultivation. It is actually a fibrous mass of

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mycelium growing on dead organic matter. Spawn may be obtained either by tissue culture method or by pure culture from germinating spores obtained from desired mushroom fruit body.

The entire process of preparation of spawn should be aseptic from the beginning to the end.

Spawn may be: grain spawn and straw spawn.

Grain spawn is prepared with grains of rye, sorghum or wheat cooking in water and sterilizing in autoclave for 30 minutes, inoculating with pure culture of desired mushroom and incubating at proper temperature. When the mycelium has ran over the whole surface of the grain, the spawn is ready for use.

Straw spawn is prepared with paddy straw or wheat straw cutting into suitable pieces, soaking in water for 5 to 10 minutes, mixing in 2 per cent lime and proceeding further as done in case of grain spawn. Preparation of spawn is usually done in glass bottles.

ii. Preparation of Mushroom Bed:

At first prepare a bamboo or wooden frame of suitable size, say about 1 sqr. metre. Then prepare paddy straw bundles from un-crumpled paddy straw of not more than one year old from which leafy material has been removed. Each bundle of straw weighing around 1 kg. should be tied at both ends by strings.

Clip off unequal parts of straw at both ends of each bundle. Dip 8 such bundles in water in a tank for 18 to 24 hours. Take them out from the tank and spread on the frame prepared earlier and this constitutes the first layer of the mushroom bed.

iii. Spawning of Mushroom Bed:

Spawning is the process of planting spawn on the mushroom bed materials. Remove the spawn from the glass bottles in which spawn has been prepared. Break it into small pieces by crushing and crumbling with the fingers. Spawning should be done on the first layer of straw bundles by broadcasting keeping about 4 to 6 inches margin all around the bed un-spawned.

Gram or pigeon pea powder should now be dusted over the spawned area.

Treatment of the first layer of straw is now complete. During the spawning period proper temperature and humidity should be maintained. Never let the bed surfaces dry out. As the spawn grows, it produces heat which contributes to the water loss. Water mushroom bed lightly with water sprinklet.

Prepare a second layer of 8 straw bundles as above and place on the first layer arranging the straw bundles crosswise with the first layer to facilitate proper aeration of spawn.

The second layer should be spawned and treated with gram or pigeon pea powder in the similar manner as done in the first. In all, mushroom bed of 4 layers of straw are to be arranged in one frame following exactly the same procedure so that the straw bundles are arranged crosswise. All the 3 layers of Straw should be spawned and treated with gram or pigeon pea powder in the same manner.

But the 4th layer should not be spawned or treated with gram or pigeon pea powder. It should be gently pressed and tied with a rope with the 3rd layer. Then place a polythene or plastic sheet on the mushroom bed in such a manner that it does not touch the straw bundles.

iv. Watering of Mushroom Bed:

The mushroom bed should be watered once or twice daily to keep the temperature range between 35° and 40°G and moisture suitable for the development of mushroom fruit bodies.

v. Mushroom Crop Production:

Small mushroom buttons (primordia of fruit bodies) will start appearing within 10 to 15 days after spawning. But it will take 4 to 6 weeks to get a crop of mushroom. Mushrooms appear in- rhythmic cycles which are called “flushes” or “breaks”.

vi. Picking of Mushrooms:

During picking, mushrooms should be lightly twisted so that neighboring young ones are not disturbed.

vii. Precautions of Mushroom Cultivation:

Precautionary measures of mushroom cultivation are: during cropping period suitable temperature, humidity and ventilation of

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mushroom bed should be maintained; insecticides like Linden or Gammexane powder should be sprinkled to check insect incidence; growth of any other fungus in the mushroom bed should be checked by spraying Captan and Zineb (0.2 per cent); and during picking of mushrooms immature fruit bodies should not be touched.

Poor cropping of mushrooms and production of abnormal fruit bodies may be at times due to virus infection. Symptoms like watery stipe and die-back are also produced as a result of virus infection.

3.9 VALUE ADDED FOOD PREPARATION FROM MUSHROOM

Aim: To understand the innovations and nutraceutical benefits of mushrooms as well as to develop interest regarding the edible mushrooms and value-added products.

Introduction:

The focus of Indian mushroom industry is predominantly on trade of the fresh produce rather than the real value-addition. Almost entire domestic trade is in the fresh form while most of the export is in the preserved form (canned or steeped). Current era is characterized by greater awareness about quality and, above all, with the demand for the readymade or ready-to-make food products. As mushrooms contain high moisture and are delicate in texture, these cannot be stored for more than 24 hours at the ambient conditions of the tropics. This leads to weight loss; veil opening, browning, liquefaction and microbial spoilage of the product making it unsaleable. Effective processing techniques will not only diminish the post-harvest losses but also result in greater remuneration to the growers as well as processors. Value can be added to the mushrooms at various levels, right from grading to the readymade snacks or the main-course item. Improved and attractive packaging is another important but totally neglected area in mushrooms- it is still unprinted plain polypouches whereas attractive and labelled over-wrapped trays are in vogue in the developed countries. Real value-added product in the Indian market is the mushroom soup powder.

Technologies for production of some other products like mushroom based biscuits, nuggets, preserve, noodles, papad, candies and readymade mushroom curry in retort pouches have been developed but are yet to be popularized. Attractive packaging of the value-added products is yet another area which may be called the secondary value-addition. While small growers may add value by grading and packaging, industry may go for the processed products for better returns as well as improvement in the demand, which shall have cascading positive effect on the production.

A. Mushroom soup powder:

Soups are commonly used as appetizers but also as main course by the diet-conscious. To prepare good quality ready-to-make mushroom soup powder using quality mushroom powder produced from the button mushroom and oyster mushroom dried in the dehumidifying air cabinet-drier.

Dried button mushroom slices or whole oyster mushrooms were finely ground in a pulveriser and passed through 0.5 mm sieve. Mushroom soup powder is prepared by mixing this powder with milk power, corn flour and other ingredients. The mushroom soup powder can be made by using the vacuum concentrated whey, a byproduct of dairy industry.

Mushroom Biscuit:

Delicious and crunchy mushroom biscuits were prepared by using the button/oyster mushroom powder and various ingredients viz., maida, sugar, ghee (bakery fats), mushroom powder, coconut powder, backing soda, ammonium bicarbonate and milk powder. For making biscuits entire ingredients were finely ground using Electric Mixture and cleaned with the help of fine sieve separately. The ingredients viz., ghee and sugar were well mixed for 5-7 minutes using Dough kneeder to make the mixture homogenous. These ingredients were added to dough kneeder for dry mixing of 20-25 minutes. Thereafter, 500 ml water was added to kneeder to make dough cohesive and homogenous and continued for next 10-15 minutes. After that dough was kept for 10 minutes under the wet cloths to make it cool. Thereafter, thin sheets of dough (1.25 cm thick)

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were made and cut into different shapes of biscuits using different steel dies. These raw cut biscuits were kept in the steel trays in systematic manner and then these trays were shifted to hot oven (180C) for baking purpose. After 20 minutes, baking trays were removed from the hot oven and after cooling the biscuits were ready to packaging and serve. The ingredient like sugar gives desired sweetness, ghee gives smoothness and ammonium bichromate gives the crunchiness to the biscuits.

Mushroom nuggets:

Nuggets are generally prepared out of „pulse" powder namely, black gram powder, soybean powder, urad dhal powder *etc*., and used in the preparation of vegetable curry in North India. The nuggets add taste as well as nutrients to the meal, since it is prepared from pulse powder. For preparation of mushroom nuggets, mushroom powder (dried and coarsely ground mushrooms) is mixed with the „Urad" dhal powder and a paste is prepared by adding required quantity of water. Ingredients and spices are added to the prepared paste and round balls of 2-4 cm diameters are made out of the paste. The prepared balls are spread over a steel tray and are dried by sun-drying method and thus the mushroom nuggets are prepared. These nuggets can be relished in two ways: straightaway this can be deep-fried and used as snacks or can be used in vegetable curry preparation along with suitable vegetables or alone.

Mushroom candy:

A fruit or vegetable impregnated and coated with sugar, subsequently taken out and dried is called a candied fruit or vegetable. The process for making candy is practically the same as that employed in the case of mushroom preserve described elsewhere, with the difference that the produce is impregnated with a higher concentration of sugar. The total sugar content of the impregnated produce is kept at about 75% to prevent fermentation.

Fresh mushrooms after harvesting are washed and halved longitudinally into two pieces. Halves are blanched for 5 min in 0.05% of KMS solution. After draining for half an hour these are treated with sugar. Sugar treatment is given at the rate of 1.5 kg sugar per kg of

blanched mushroom. Initially sugar has to be divided into three equal parts. On the first day, blanched mushrooms are covered with one part of sugar and kept for 24 h. Next day, the same mushrooms are covered with second part of sugar and are kept overnight and on the third day mushrooms are removed from the sugar syrup. The sugar syrup is boiled with 3rd part of sugar and 0.1% of citric acid to bring its concentration up to 70°Brix. Mushrooms are mixed with this syrup and again the contents are boiled for 5 min to bring its concentration upto 72° Brix. After cooling, the mushrooms are removed from the syrup and drained for half an hour. The drained mushrooms are placed on the sorting tables to separate, to reject defective and unwanted pieces. Finally mushroom pieces are subjected to drying in a cabinet dryer at 60°C for about 10 h. As soon as these become crispy, all mushrooms are taken out, packed in polypropylene bags and sealed. The mushroom candy can be stored up to 8 months with excellent acceptability and good chewable taste.

Mushroom preserve (Murabba):

Murabba (preserve) is made from matured fruit or vegetable, by cooking it whole or in the form of pieces in heavy sugar syrup, till it becomes tender and transparent. In murabba preparation, around 45 kg of fruit or vegetable is used for every 55 kg of sugar and cooking is continued till a concentration of at least 68% of soluble solid is reached. Fresh button mushrooms are graded, washed, pricked and blanched in 0.05% KMS solution for 10 min. It is treated with 40% of its weight of sugar daily for 3 days. Then, mushrooms are taken out from the syrup and 0.1% citric acid and remaining 40% of sugar is mixed in the syrup. After bringing its concentration to 65Brix, mushrooms are added in the syrup and good quality murabba is prepared.

Mushroom Pickle:

Mushrooms for pickling are either blanched or fried in oil till brown depending upon taste; various condiments as per local preferences and practices are also ground or fried in oil separately and added to the mushroom. The contents are mixed thoroughly and cooked slightly for few minutes. It is allowed to cool and then filled in the jars (lugs) of

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desired size. Vinegar may be added for taste and longer storage and the contents in the bottle or the container should be topped up with oil.

According to a formulation developed and standardized at the DMR and its coordinating centers, mushrooms are washed, sliced and blanched for 5 min in 0.05% KMS solution. The blanched mushrooms are washed in cold water for 2-3 times and the excess water is drained off. Then the mushrooms are subjected to salt curing process, in which 10% sodium chloride is added and kept overnight. The excess water oozed-out of mushroom is removed on the next day and spices & preservatives are mixed to the desired taste and quality of mushroom pickle. The various spices namely turmeric powder, black mustard seed powder (rai), red chilly powder, cumin seed powder, fenugreek seed powder, aniseed powder (suwa/ shopa), black pepper, carom seed (ajwain), nigella seed (kalonji), fennel seed powder (saunf) and mustard oil are added to prepare tasty pickle. Acetic acid and sodium benzoate within the permitted limits are used as preservatives.

This pickle can be stored upto one year in the lug bottles. Good quality pickle can also be prepared from oyster mushroom. Cleaned mushrooms are blanched in hot water (80^{o}C for 5 min), rapidly cooled and added to 60% brine to obtain mushroom to brine ratio of 7:3 by volume. The mixture is maintained at 15-20^{o}C for 15 days for fermentation and further kept at 0-4^{o}C to obtain a pH of 3.9. Sugar is added to the preparation at the rate of 3.3% by weight to the brine and final salt concentration reached to 6.6 per cent by weight. Studied on the suitability of Pleurotus spp. for pickle preservation reported that the product could be stored for a minimum period of 6 months at the ambient temperature (22-34^{o}C) without any off flavour. The pickle prepared from paddy straw mushroom having also good keeping quality.

Mushroom chips:

The freshly harvested button mushrooms are washed, sliced (2 mm) and blanched in 2% brine solution. The mushrooms are dipped overnight in a solution of 0.1% of citric acid + 1.5% of NaCl + 0.3% of red chilly powder. After draining off the solution, the mushrooms are

subjected to drying in cabinet dryer at 60oC for 8 h. Then it is fried in the refined oil and good quality chips are prepared. Garam masala and other spices can be spread over the chips to enhance the taste. After spices mixing, the chips are packed in polypropylene packets and sealed after proper labelling.

Ready-to-serve mushroom curry

In view of the growing market for the readymade / ready-to-eat food items and keeping in mind the popularity of the Indian Curry world over, a technology was developed at DMR, Solan for production of "Mushroom curry in flexible-retortable pouches". Flexible retort pouch is an ideal alternate to metal containers for packing and storage of heat processed foods. Flexible retort pouches, besides being cheaper, have many other advantages-easy bulk packing and transport, sale and very convenient to the end-user. The retort pouch of 105 um thick with polypropylene outer layer (80 μ), aluminium middle layer (12.5 μ) and polyester inner layer (12.5 μ) available in the market was used for packing mushroom curry.

In a frying pan, oil was added and heated. Sliced onions and green chillies were added to the oil and fried till golden brown. Garlic and ginger were ground into a paste, added and lightly fried till oil reappeared. Curry powder, salt and red chilly powder were added and lightly fried. About one litre of water was added to the spices mixture and boiled till thick consistency was obtained. Hundred grams of cut mushrooms were filled in the retort pouch and 50 g of curry was added into the pouch. Then it was heat processed for FO value of 10 (final 13.2) at 121C for 43 min and cooled rapidly. The ready-to-serve mushroom curry prepared was delicious with good taste, attractive colour and a storage life of one year. Mushroom curry was also successfully prepared from dried oyster mushroom and button mushroom after its rehydration.