Practical physiology of fungi

For 4th year of B.Sc. students

Prepared by

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Culture media

Classification of culture media

Criteria used for classifying culture media includes their chemical composition, physical properties and their use. Every culture medium is designed for a definite use and hence its physical and chemical characteristics depend on its application and function.

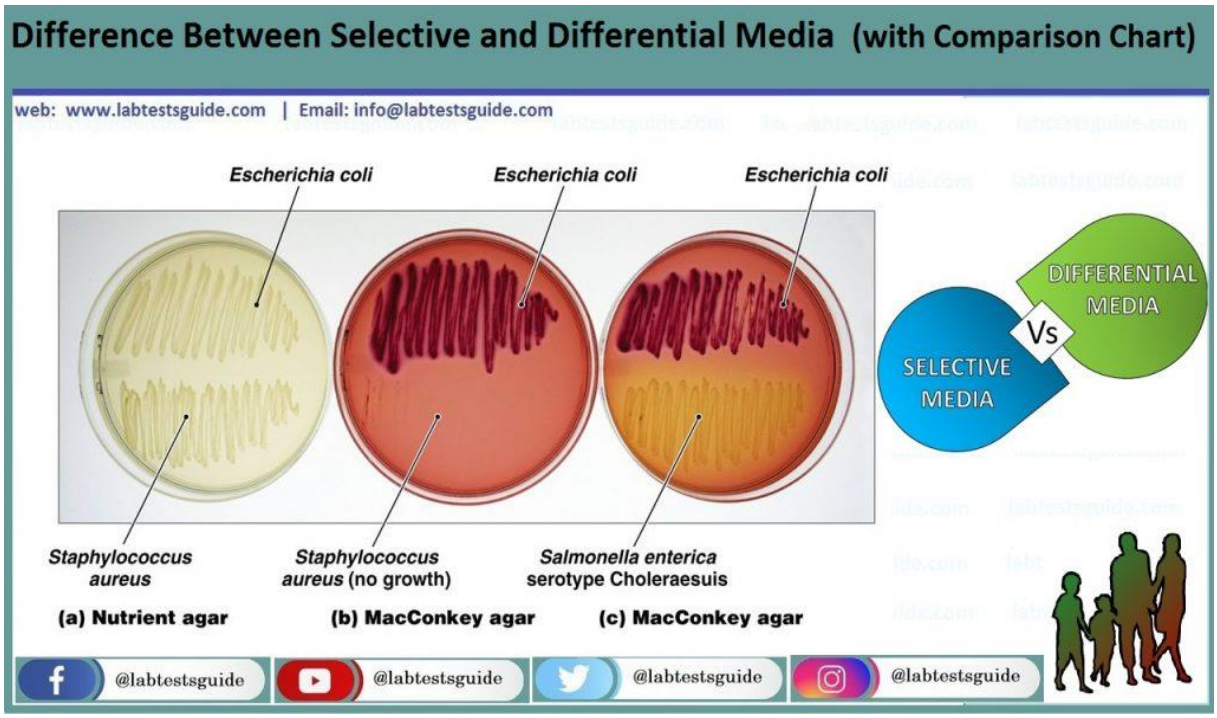
I- Classification of culture media according to their use

According to their use culture media are divided into the following types:

1- **Routine laboratory media**: These media contain certain complex raw materials of plant or animal origin such as yeast extract, malt extract, peptone etc., and are employed for routine cultivation and maintenance of a wide variety of fungi.

2- Enriched media: These media are prepared by supplementing the routine laboratory media with some specific substances such as vitamins and amino acids to meet the nutritional requirements of more fastidious of fungi and are employed for their cultivation.

3- Selective media: These media facilitate the isolation of a particular group or species of microorganisms from mixed cultures. Such media contain substances which inhibit microorganisms except the desired group or species.



4- Differential media: These media are supplemented with certain reagents or chemicals for differentiating between various kinds of microorganisms on the basis of visible differences in their growth patterns. Such type of media is used more often in bacteriological studies.

5- Assay media: These type of media is specifically employed for the assay of some metabolites such as enzymes, vitamins, amino acids, antibiotics, disinfectants etc., and are of definite composition.

6- Biochemical media: These media are generally used for the differentiation of microorganisms on the basis of their biochemical activities, and are helpful in the study of their metabolic processes.

II- Classification of culture media according to their chemical composition: According to their chemical composition media are classified into the following types:

1- Natural media: The natural medium comprises entirely complex natural products of unknown composition. The raw material of a natural medium may be

of plant or animal origin, and some of the common ingredients employed for this purpose include extracts of plant and animal tissues, e.g., fruits, vegetables, egg, milk, blood, body fluids, yeast, malt and manure extracts etc. Obviously, the chemical composition and concentration of a natural medium is not well defined. On account of their complex nature, these media are able to support a variety of organisms, and hence are quite useful for routine laboratory cultures of fungi.

2- Semisynthetic media: These media are so designed that some of their constituents are of known chemical composition, while others are derived from some natural sources with unknown composition. The chemical composition of a semisynthetic medium is partly known. The medium is a best serve as a routine medium and sometimes for physiological studies. Potato dextrose agar (PDA) is one of the popular media.

3- Synthetic media: These are chemically defined media of known composition and concentration. The media are exclusively composed of pure chemical substances. However, absolute purity of the ingredients is achieved, although substances of only analytical reagent quality are used for such purposes. One account of their known composition as well as being in solution, these media are quite useful for nutritional and metabolic studies of fungi. The composition of these media may be amended as per requirement and as such they may be simple or complex in composition. A simple synthetic medium contains a single carbon and energy source, a nitrogen source, generally as ammonium salt, some Sulphur and phosphorus sources and various minerals. All these ingredients are dissolved in a buffered aqueous base. However, for more fastidious organisms, a complex synthetic medium is designed by incorporating some additional factors such as certain vitamins, amino-acids, purines, pyrimidines etc., or by employing a multitude of carbon and nitrogen sources together.

C- According to their physical states: Media are classified into the following types:

1- Solid media: Media in solid state are in use since the beginning of laboratory studies of fungi. The first laboratory culture of fungi was obtained on a solid media such as fruit slices. Some common examples of such media are nutrient impregnated slices of potato, carrot, sugar-beet etc. and coagulated egg or serum. However, with the advent of agar as a solidifying agent, such media have largely been replaced by agar media. Use of fruits and vegetable slices in the cultivation of fungi is now more or less restricted to the baiting technique employed for isolation of some specific organisms.

2- Solid-reversible to liquid media: Such reversible media were first introduced by Koch (1881) who observed that addition of 2 to 5 percent of gelatin to the commonly employed media rendered them a semi-solid consistency. However, gelatin could not find a wide application on account of its low melting point (37˚C), and also because it is hydrolyzed by many proteolytic bacteria at ordinary temperature. The use of agar for solidifying culture media was also initiated the same year and in the same laboratory.

3- Semi-solid media: These are media with gelatinous consistency and are employed for specific purpose. They contain a small amount of agar or some other solidifying agent like corn meal. These media are sometimes used for the study of motile reproductive structures of fungi.

4- Liquid media: These are media without any solidifying agent, and are indispensable for most of the quantitative studies of fungi. Nutritional and metabolic studies of fungi, as well as microbiological assays are invariably carried on liquid media. Some of the advantage of liquid media is that they permit the cultures to be aerated, the mycelium to be weighed and the metabolic products to be analyzed easily. However, with respect to routine studies, liquid media have some distinct disadvantages. Growth in liquid media does not manifest the morphological characteristics of microorganisms. They are also difficult to handle

without disturbing the culture. Moreover, liquid media are least helpful in the purification of microorganisms from a mixed culture. For an even distribution of nutrients and for providing uniform aeration to growing fungus, the liquid cultures are sometimes put to constant mechanical shaking.

Sterilization

Sterilization refers to the process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses and spore forms etc.) from a surface, equipment, foods, medications, or biological culture media.

Sterilization can be achieved through application of heat, chemicals, irradiation and filtration.

There are three main methods for sterilization:

1- Physical methods

2- Chemical methods

3- Mechanical methods

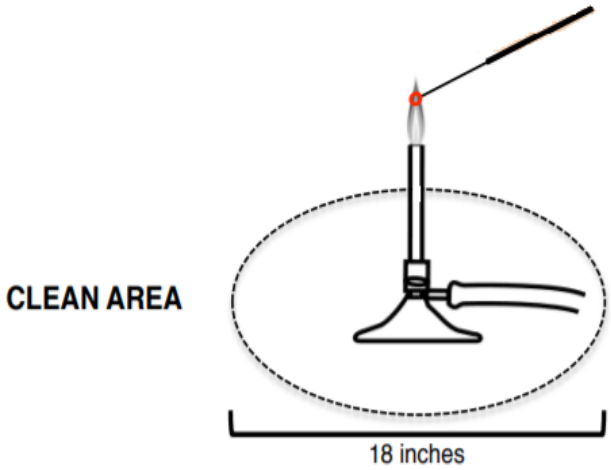
1- Physical methods

Sterilization by heat

Heat may be utilized for sterilization either in dry or moist form. However, moist heat is much more effective and requires both shorter duration and lower temperature. Sterilization by moist heat generally is complete at 121°C for 15-30 minutes of exposure. On contrast, sterilization by dry heat requires a temperature of 160°C for 60 minutes. The two kinds of heat treatments kill the microorganisms by coagulating and denaturing their enzymes and other proteins.

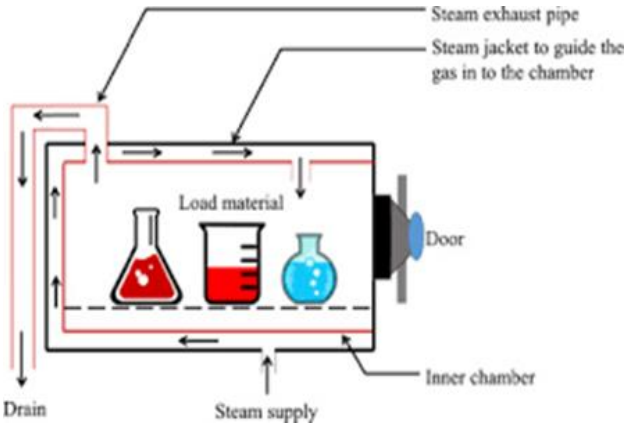
Application of dry heat

a- Flaming



b- Hot-air oven

Oven



radiation

c- Radiation (Infra-red or Ultra violet)

Application of moist heat

The use of the Autoclave for sterilization

2- Chemical methods

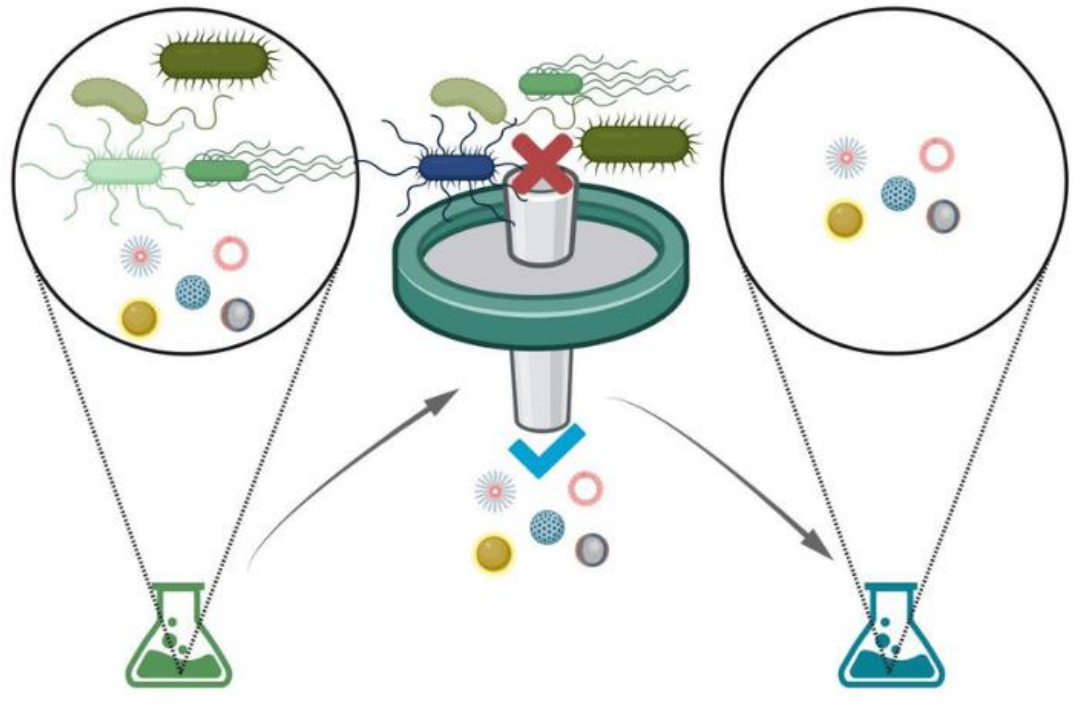
Using of chemical substances as agents, like chloroform, mercuric chloride, formaldehyde and ethyl alcohol.

3- Mechanical methods

Sterilization by filtration

This technique employs special type of filters having pores so small that ordinary bacteria are arrested. This method is particularly useful for sterilizing heat sensitive materials, such as culture media containing serum, antibiotic solutions, culture filtrates etc. The most common filters are Seitz filters and Cellulose membrane filters.


Flask of sample
Cap
Membrane filter
Cotton plug in vacuum line ensures sterility
Sterile filtrate
Vacuum line



Methods for measuring fungal growth

Growth may be defined as the orderly increase in cell components leading to an increase in biomass (Prosser, 1995). The growth form of filamentous fungi is complex; extension of individual hyphae is localized at the tip, whereas biomass synthesis supporting that growth may take place throughout the mycelium. The growth of a fungus can be measured in various ways such as increase in colony diameter, increase in dry weight, rate of production of different type of metabolites, etc. The mechanisms involved in the control and regulation of mycelial growth are better studied on solid medium than in submerged cultures, as fungi are adapted to growth on solid substrates.

Direct methods

Some of the direct methods to determine fungal growth are stated afterwards:

Growth of hyphal extension

Measure of hyphal extension rate is measured microscopically on solid medium as an increase in length, e.g. measure of the increase in radii of circular colonies, daily or every other fixed period of time, after inoculating the mold in an agar plate and incubate it at the appropriate conditions. This is probably the most common technique for estimation of growth of filamentous fungi on solid media. A growth rate function can be derived by plotting colony diameter against time and measuring the slope of the straight part of the line. Analysis is now greatly facilitated by the increased availability of image analysis systems which enable automated measurement of hyphal lengths and subsequent kinetic analysis of data (Wiebe and Trinci, 1990; Gray and Morris, 1992).

Measure of fungal biomass

Molds are usually grown on the surface of a cellophane membrane, overlaying the agar, from which the biomass can be washer or otherwise removed for the determination of the dry weight. This measure is also possible in liquid cultures. In both solid and liquid media, separation of biomass from the growth medium is slow, tedious and requires relatively large amounts of biomass for accuracy.

Turbidimetric techniques are less reliable due to the heterogeneous nature of liquid cultures of filamentous fungi.

Indirect methods

Ergosterol

Fungal plasma membranes are similar to mammalian plasma membranes, differing in having the nonpolar sterol ergosterol, rather than cholesterol, as the principal sterol. The plasma membrane regulates the passage of materials into and out of the cell by being selectively permeable. Membrane sterols provide structure, modulation of membrane fluidity, and possibly control of some physiologic events. Fungal growth and biomass could therefore be estimated by measuring this specific component of fungi. Quantifying ergosterol production in foods has proved more difficult. Since now, ergosterol content has been mainly assayed in cereal samples. The determination of ergosterol is also valuable in correlating metabolites such as aflatoxins and OTA (Gourama and Bullerman, 1995; Saxena et al. 2001).

Impedimetry and conductimetry

Metabolites produced by growth of microorganisms in liquid media alter the medium's impedance and conductance. The use of changes in these properties has been used to estimate fungal growth. A major problem of these techniques involves the selection of suitable media, but when the method is set up, this method results rapid and effective.

Adenosine triphosphate (ATP)

Another measure of microbial biomass is the measure of the bioluminescence emitted by the molecules of fungal ATP. However, living plant cells contain also high levels of ATP and fungi are often very difficult to separate from food materials.

Pectinesterase

The fundament of this technique is that gas liquid chromatography is used to determine the amount of methanol released from pectin by the fungal enzyme

pectinesterase. This is considered a rapid method for detecting viable spores of spoilage fungi, but it needs some improvements before practical application.

Fungal volatiles

It consists in measuring the effects of fungi on foods. Fungi produce chemical volatiles during growth and particular chemicals can be detected and therefore measure fungal growth in an indirect way. Several commercial gas sensor array instruments are now available on the market covering a variety of chemical sensor principles, system design and data analysis techniques. A series of different detection principles can be used in chemical gas sensors: heat generation, conductivity, electrical polarization, electrochemical activity, optical properties, dielectric properties and magnetic properties. In principle, the results obtained from a gas-sensor array represent qualitative and quantitative information of the composition of the headspace gas mixture of a sample. The technique should therefore have a great potential in a number of applications related to food. Numerous **electronic nose** studies related to food already have been published, but the electronic nose technology applied on food must be regarded as being in its early stage. A goal of this technology is to explore the use of an electronic nose for rapid detection of food spoilers and pathogens via development of a standard curve of some potential volatile compounds that can be used to develop some specific aroma-labeled substrates.

Immunological techniques

Fungal cell wall proteins produce antigens, which can be detected by immunological methods. Some antigens are derived from components common to a wide range of fungi, and hence are indicative of general fungal growth, while others are genus or even species specific.

Molecular methods

They are based on nucleic acid sequences that are specific to the target fungi. The most known method is called **nucleic acid hybridization** and it involves the selection, cloning and chemical labelling of sequences specific to the target

organism. These are then used as probes to detect RNA or DNA of the pathogen in extracts of the substrate. DNA may be specific at almost any taxonomic level. In some instances the detection and identification of the causal agent(s) may be secondary to other consideration. For example, it may be more important to quantify the amount of pathogen present rather than just determine its identity. Several approaches have been taken to develop diagnostic assays, and are divided into immunological and DNA-based systems, this last generally being **polymerase chain reaction** (PCR). In contrast to hybridization, PCR-based assays for detecting mycotoxins in fungi have been widespread in the last years. PCR is an extremely sensitive technique and involves the enzymatic amplification of a target DNA sequence by a thermostable DNA polymerase.

Experiment 1

1- Isolation of fungi from natural sources

- Preparation of culture media and sterilization.
- Isolation of fungi on the suitable media for fungal growth.
- Identification and preservation of fungi

Table 1: …….

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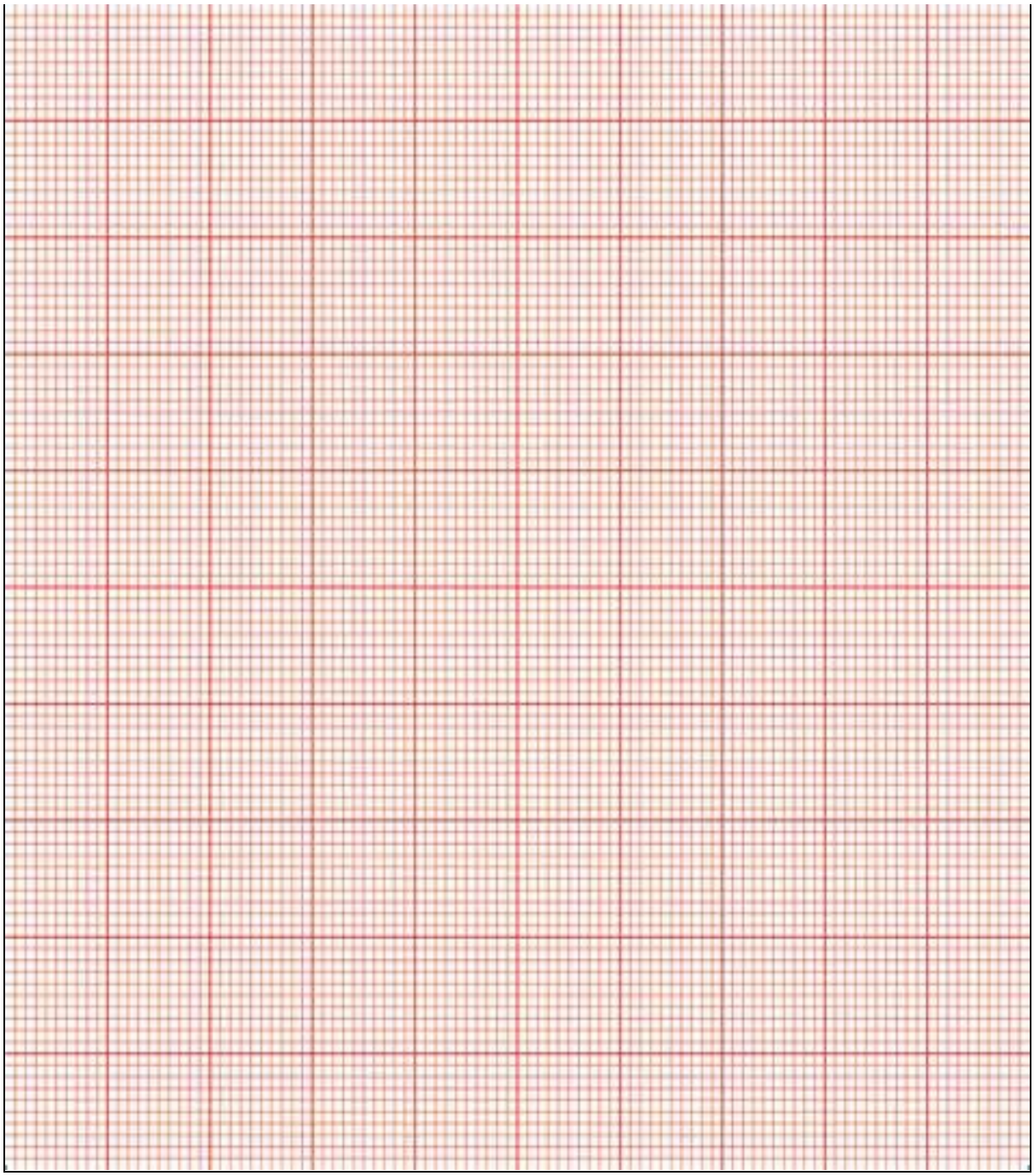
Fungi	Counts	%	OR



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2- Factors affecting fungal growth and metabolism

Fungi have in the course of evolution diversified to exploit a wide variety of habitats. Different species hence require different conditions for optimal growth. Microbial metabolism is significantly influenced by the physical and chemical environment. Thus, toxin-producing fungi may invade food at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest food, toxinogenic fungi can be divided into three groups (Suttajit, 1989).

- Field fungi, includes species of plant pathogenic fungi, usually with high requirements of water, such as *Alternaria*, *Cladosporium* and *Fusarium*.
- Storage fungi, with lower requirements of humidity, are principally the genus *Aspergillus* and *Penicillium*.
- Advanced deterioration fungi, normally do not infest intact food, but easily attack damaged one and require high moisture content. Some examples are some other aspergilli species, *Chaetomium*, *Scopulariopsis*, *Rhizopus*, *Mucor* and *Absidia*.

The main factors that influence growth of fungi include temperature, pH and moisture. But apart from environmental factors, chemical and biological factors clearly play a role (Figure 49). Under some circumstances these effects are additive. Under others, the implication is that synergistic interactions lead to a combined effect of greater magnitude than the sum of constraints applied individually. This has been described by Leistner and Rödel (1976) as the 'hurdle concept.' Moreover, hurdles are frequently combined to minimize the impact of processing on the quality and to improve the safety of ready-to-eat foods.

Environmental factors

The large and diverse group of microscopic foodborne yeasts and molds includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile, environmental requirements. There

are several major parameters governing fungal invasion, growth and production of mycotoxins:

Experiment 2

Temperature

Temperature is an important environmental factor affecting growth and mycotoxin production by molds. Fungi are capable of surviving under the full range of temperatures normally experienced in environments in which they live. The temperature range usually reported for fungal growth is broad (10-35°C), with a few species capable of growth below or above this range. Fungi can be divided according to their tolerance to temperature in psychrophilic, mesophilic, and thermophilic fungi.

Procedures

1- Preparation of culture media and sterilization

2- Cultivation of fungi

3- Incubation of cultures under various temperatures

3- At the end of the incubation period, filtration of cultures and determination of mycelial growth that grown under various temperatures.

4- Illustrate the data and write a comment.

Table 2:……..……..…

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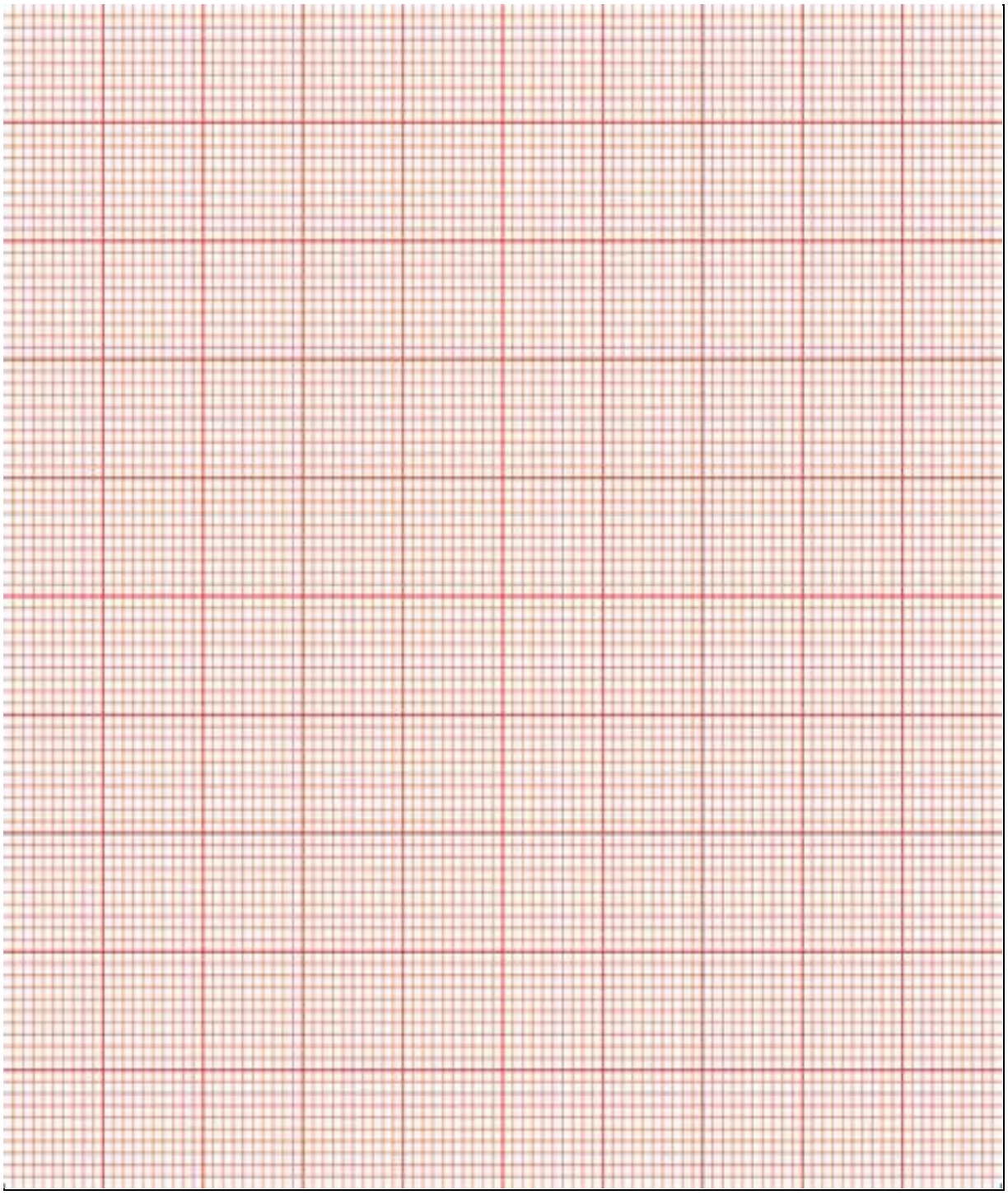
Temperature	*Aspergillus*	*Fusarium*	*Rhizopus*
15			
20			
25			
30			
35			
40			



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Experiment 3

Hydrogen ion concentration (pH)

In general, there is a lack of information on the effect of pH on fungal growth parameters, in spite of a considerable literature on growth in relation to the initial pH of media. This data is of limited value, since fungal metabolism alters pH during their evolution. Hydrogen ion concentration in a medium could affect growth either indirectly by its effect on the availability of nutrients or directly by action on the cell surfaces. The acid/alkaline requirement for growth of all yeasts and molds is quite broad, ranging from pH 3 to above pH 8, with optimum around pH 5, if nutrient requirements are satisfied. In general, Aspergillus species are more tolerant to alkaline pH while *Penicillium* species appear to be more tolerant to acidic pH (Wheeler et al., 1991). It is seen that in situations near neutral pH, fungi must compete with bacteria for niches, and at higher aw values most fungi are not competitive in mixed culture. However, where a w is below 0.90, fungi become dominant irrespective of pH. In specialized niches where bacteria do not appear to have a role as pathogens, specific Fusarium and *Penicillium* species are dominant even at neutral pH and high a_w (Wheeler et al., 1991).

Procedures

1- Preparation of culture media with variable pH values and sterilization

2- Cultivation of fungi

3- Incubation of cultures under suitable temperature

3- At the end of the incubation period, filtration of cultures and determination of mycelial growth that grown under various pH values.

4- Illustrate the data and write a comment.

Table 3:……..……..…

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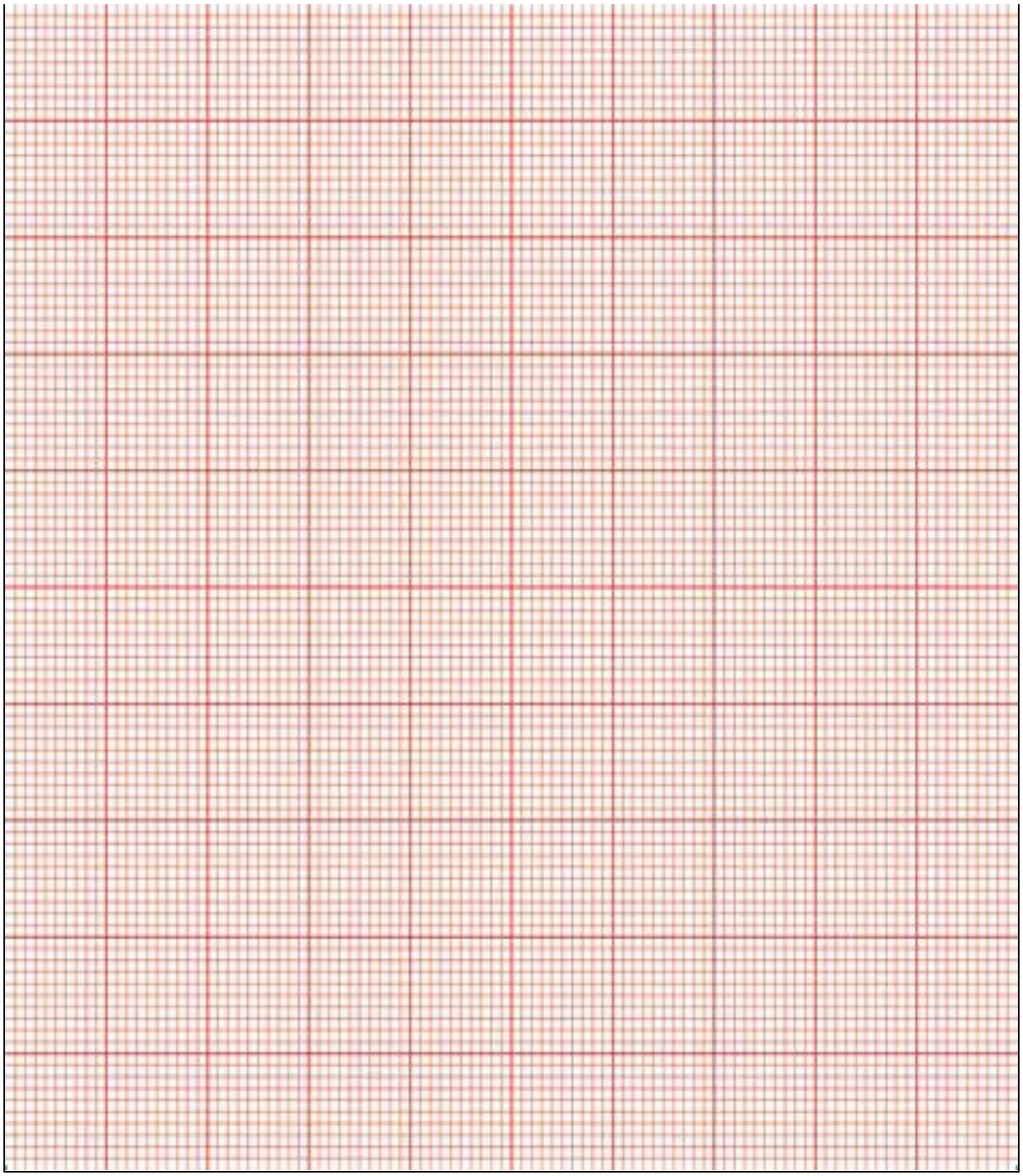
pH values	*Aspergillus*	*Fusarium*	*Rhizopus*
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			



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Availability of water

Living organisms consist largely of water. Hence if an organism is to grow has to take up water from the environment. Whether water enters or leaves a cell depends on the difference between the water potential of the cell and that of the surrounding medium, water moving from a region of high to one of lower water potential (Carlile and Watkinson, 1996). In microbiology, three measures of the water availability have been used: water potential (ψ), water activity (a_w), and relative humidity (R.H.).

Water potential is the sum of numerous components, of which the most important are osmotic, matric and turgor potential, and is measured in units of pressure.

Water activity is a fundamental property of aqueous solutions, and by definition is the ratio of the vapour pressure of the water in the substrate (P) to that of pure water at the same temperature (P0):

$$a_w = P/P0$$

p0 p aw = Water activity ranges from zero (water absent) to 1.0 (pure water). For an ideal solution aw is independent of temperature, and in actual practice, the aw of a given solution varies only slightly with temperature within the range of temperature permitting microbial growth. The relationship between water potential and water activity is given by the next equation, where the value of k depends on temperature and is, for example, 1.37 at 25ºC and 1.35 at 20ºC.

$$\psi \text{ (Mpa)} = k \ln a_w$$

Not only is the availability of water in the surrounding liquid phase of importance to fungi, but the water content of the adjacent gas phase. The water content of the atmosphere is expressed in terms of **relative humidity**, the ratio of the water vapour pressure of the gas phase being considered, to that of a saturated atmosphere at the same temperature. It is hence the same ratio as water activity but expressed as a percentage. In most of the studies presented in this thesis, aw was used to describe the status of the water in solution or substrate in preference

to R.H., which applies more strictly to the surrounding atmosphere. Under equilibrium conditions the two terms are interchangeable. A required aw in the environment of a fungus may be obtained either by fixing the water content or the solute concentration in the culture substrate or by keeping the substrate in equilibrium with an atmosphere of controlled R.H. Moisture requirements of foodborne molds are relatively low; most species grow at a 0.85 a w or less, although yeasts generally require a higher water activity. 0.60 aw is considered the limit for cell growth, but spores of Aspergillus and *Penicillium* for example, are able to survive at lower aw for several years (Carlile and Watkinson, 1996). Moisture control is the best and most economical means to control the environment to prevent mold growth and mycotoxin production.

Light

There are some reports that illumination will increase or more commonly reduce the rate at which fungi spread across an agar surface. Such effects are sometimes due to the photochemical destruction of components of the medium but in other instances a direct effect on metabolism seems likely. The biosynthesis of pigments, mainly carotenoids, as consequence of light action has been demonstrated.

Availability of oxygen

Organisms can obtain energy by oxidative (respiratory) metabolism or by fermentation. The implications for oxygen requirements of the occurrence of respiration, fermentation or both in a fungus divided them in obligate aerobes, facultative anaerobes and obligate anaerobes (Carlile and Watkinson, 1996): Food spoilage molds, like almost all other filamentous fungi and yeasts, have an absolute requirement for oxygen. However, many species appear to be efficient oxygen scavengers, so that the total amount of oxygen available, rather than the oxygen tension, determines growth. The concentration of oxygen dissolved in the

substrate has a much greater influence on fungal growth than atmospheric oxygen tension (Pitt and Hockings, 1997). The most oxygen demanding molds will colonize the surface of the food, while the less exigent could be found inside the food. Although probably not economically feasible, one sure way to prevent mycotoxin contamination of cereals and other food, is to store them under anaerobic conditions, e.g. CO_2 or nitrogen. For instance, this could be done in large airtight silos. The molds would not grow, but this type of environment control is sometimes understandably unrealistic.

Chemical factors (**Nutritional factors**)

Experiment 4

Effect of different Carbon sources on fungal growth

All forms of life, including molds, require exogenous materials to build into biomass. As heterotrophs, the molds require organic compounds for both the synthesis of biomass (anabolic metabolism) and to produce the energy to drive these reactions (catabolic metabolism). These aspects of metabolism are frequently referred to as primary metabolism (Smith and Moss, 1985). Fungi can use a number of different **carbon sources** to fill their carbon needs for the synthesis of carbohydrates, lipids, nucleic acids and proteins. Oxidation of sugars, alcohols, proteins, lipids, and polysaccharides provides them with a source of energy. Differences in their ability to utilize different carbon sources, such as simple sugars, sugar acids, and sugar alcohols, are used, along with morphology, to differentiate the various yeasts.

Procedures

1- Preparation of culture media with different carbon sources and sterilization

2- Cultivation of fungi

3- Incubation of cultures under suitable temperature and pH

3- At the end of the incubation period, filtration of cultures and determination of mycelial growth that grown under different carbon sources.

4- Illustrate the data and write a comment.

Table 4:……..……..…

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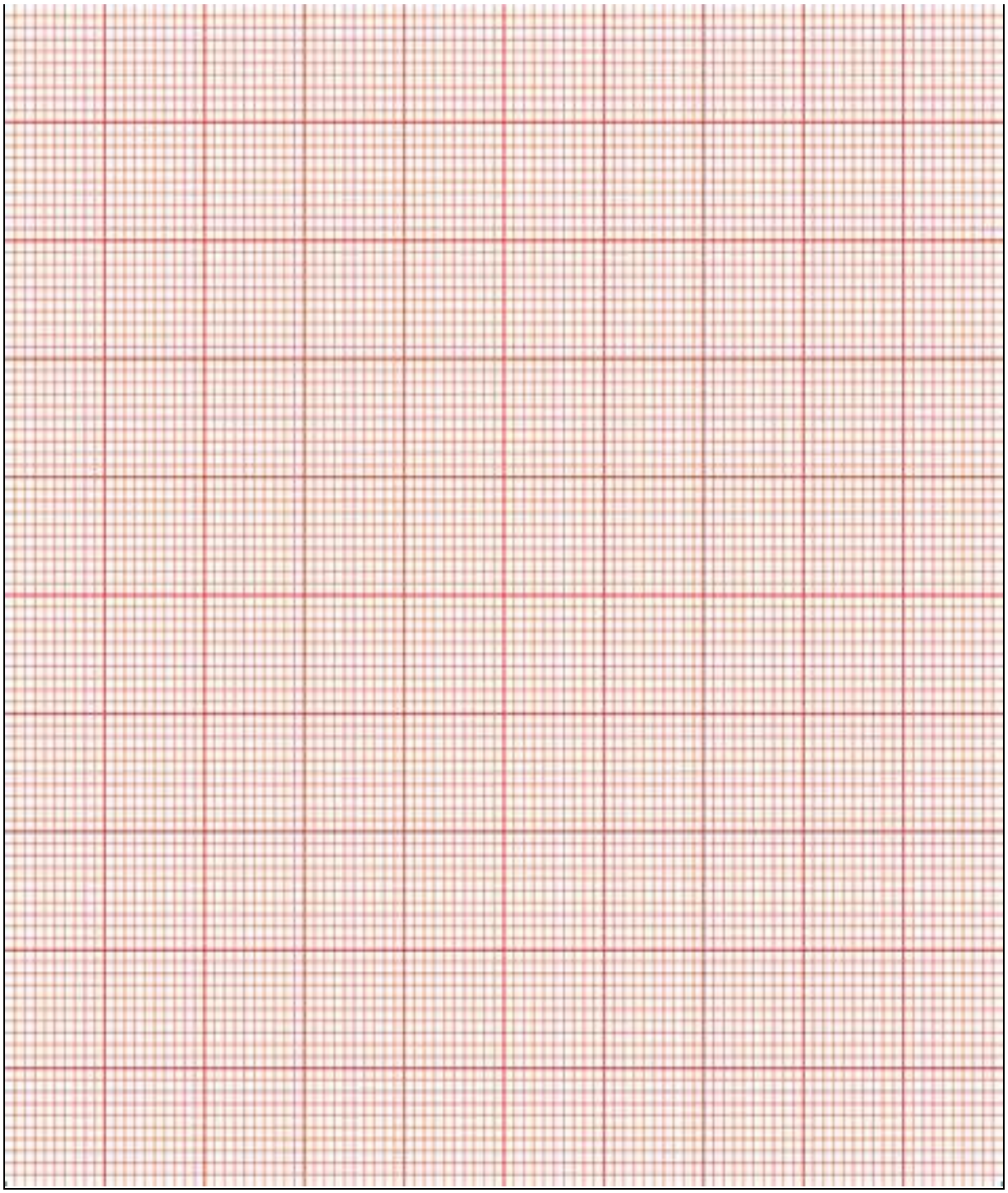
Carbon sources	*Aspergillus*	*Fusarium*	*Rhizopus*



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Experiment 5

Effect of different Nitrogen sources on fungal growth

Fungi require a source of **nitrogen** for synthesis of amino acids for proteins, purines and pyrimidines for nucleic acids, glucosamine for chitin, and various vitamins. Depending on the fungus, nitrogen may be obtained in the form of nitrate, nitrite, ammonium or organic nitrogen as no fungus can fix nitrogen. Most fungi use nitrate, which is reduced first to nitrite and then to ammonia. Therefore, availability and type of nutritional factors such as carbon source and nitrogen source can also affect both mycotoxin production and morphological differentiation. Other major nutrients for fungi are sulphur, phosphorus, magnesium and potassium, which can be supplied to most fungi as salts. Trace elements like iron, copper, manganese, zinc and molybdenum are required by nearly all fungi as cofactors for enzymes. But in high amounts, some trace elements can become toxic for some fungi. For example, OTA production by *A. ochraceus* strains varied with the different concentrations of yeast extract (0-4%) and sucrose (0-4%) in a laboratory medium (Atalla and El-Din, 1993).

Procedures

1- Preparation of culture media with different nitrogen sources and sterilization

2- Cultivation of fungi

3- Incubation of cultures under suitable temperature and pH

3- At the end of the incubation period, filtration of cultures and determination of mycelial growth that grown under different nitrogen sources.

4- Illustrate the data and write a comment.

Table 5:……..……..…

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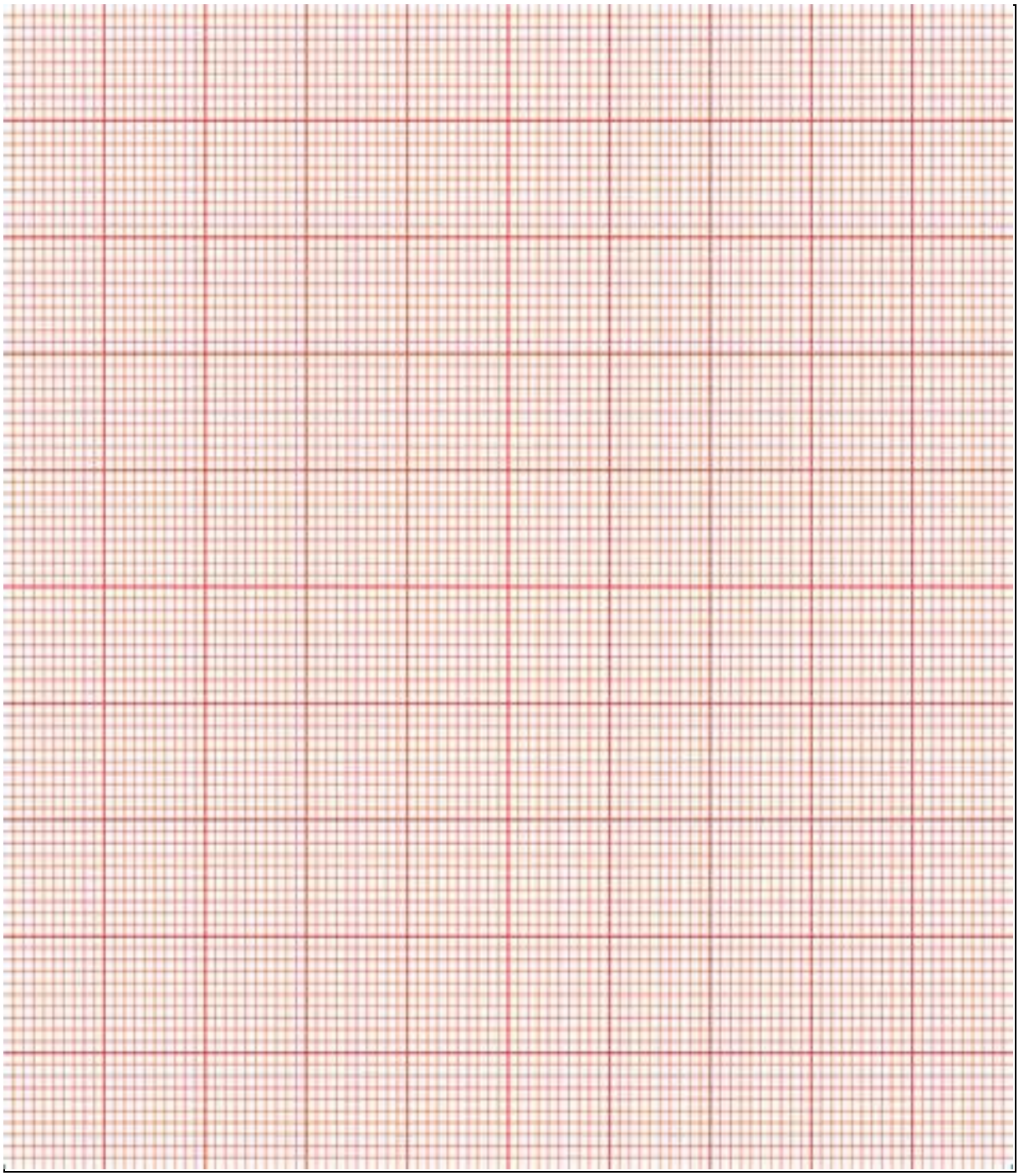
Nitrogen sources	*Aspergillus*	*Fusarium*	*Rhizopus*



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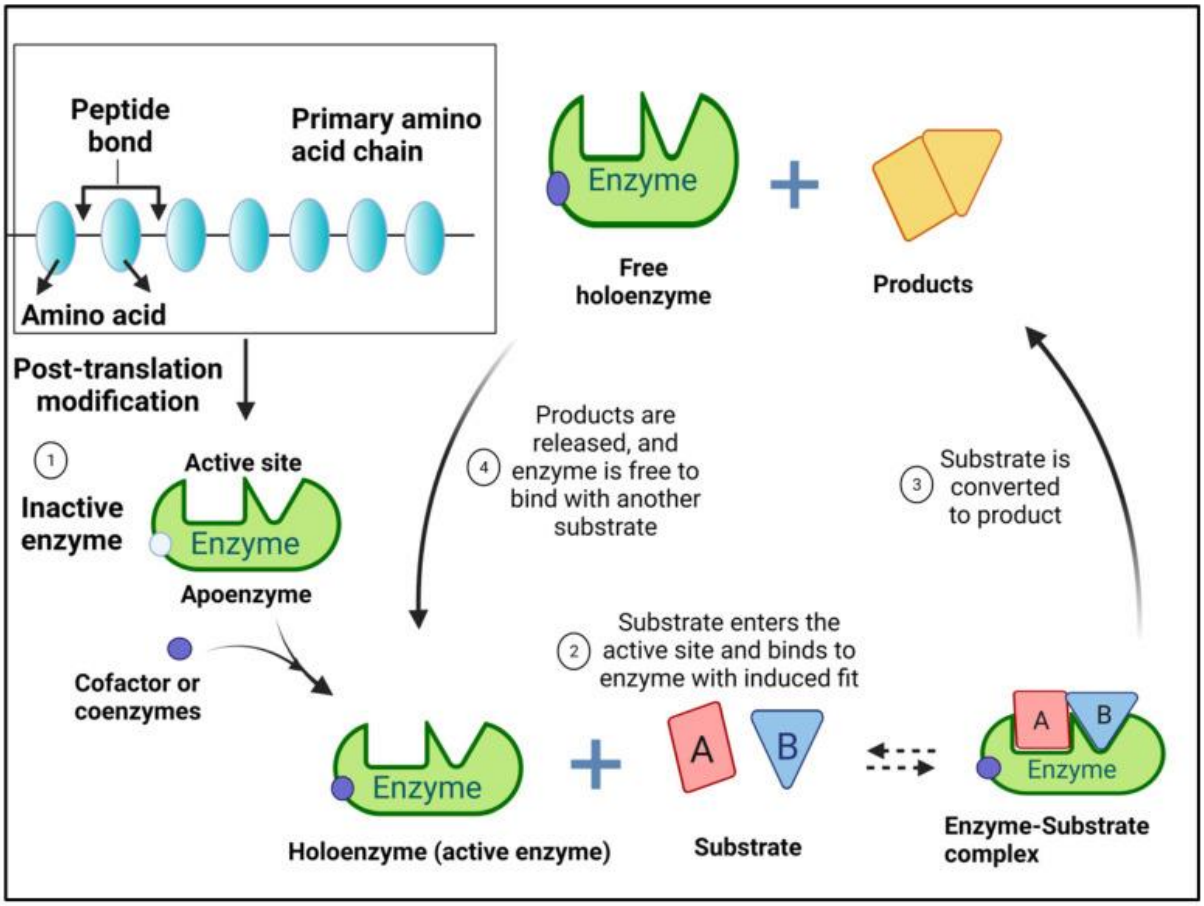
Experiment 6

Fungal enzymes

Enzymes have played an important role in different types of biological systems for various applications. They are proteins that break down and convert complicated compounds to simple products. Fungal enzymes are compatible, efficient, and proper products for many purposes such as medicinal uses, industrial processing, bioremediation process, and agricultural applications. Fungal enzymes have been used in many industries, including baking, brewing, cheese making, antibiotics production, and commodities manufacturing, such as linen and leather. Furthermore, they also are used in other fields such as paper production, detergent, the textile industry, and in drinks and food technology in products manufacturing ranging from tea and coffee to fruit juice and wine. Recently, fungi have been used for the production of more than 50% of the needed enzymes. Fungi can produce different types of enzymes extracellularly, which gives a great chance for producing in large amounts with low cost and easy viability in purified forms using simple purification methods. Hydrolases are the most extensively studied groups of enzymes; they catalyze the hydrolysis of their substrate through the addition of water. Hydrolases represent the most commercially marketed enzymes due to their wide application in different industrial sectors. Fungal amylases, proteases, lipases, and cellulases represent the most commercially demanded enzymes.



Schematic illustration for enzyme structure, activation, and steps of enzyme and substrate interaction.

Amylases

Amylase enzymes are used for commercial application and was firstly applied medicinally in treating digestive disorders. Amylases could be classified into α, β, and γ-Amylases depending on the attaching site in the starch molecules and the nature of the resulting products. α-Amylases are calcium-dependent metalloenzymes that act randomly on the starchy substrates yielding maltose and maltotriose from amylose or glucose and dextrin from amylopectin. β-Amylases hydrolyze 1,4-glycosidic bonds in the carbohydrate chain, yielding one maltose unit. They are extensively important in plants, especially in the seed ripping process, but they are also reported from the microbial origin. γ-Amylases resemble the other two types of amylases in hydrolysis activity toward 1,4-

glycosidic linkages, unlike the two forms characterized with 1,6-glycosidic linkages hydrolysis activity and preferring acidic environment pH 3. *Aspergillus niger* is considered the potent commercial α-Amylase producer among all filamentous fungi. Many other fungi were reported for their capacity to produce different types of amylases, including *Aspergillus oryzae*, *A. terreus*, *Fusarium solani*, and *Penicillium citrinum*.

Lipases

Lipases are a group of hydrolytic enzymes that act by hydrolysis of triacylglycerol yielding fatty acid and glycerol. Lipases also catalyze the reverse reaction by esterification of glycerol and fatty acid. Fungal lipases are produced by several fungi including *Aspergillus niger*, *Penicillium verrucosum*, *Fusarium solani*, *Arthrographis curvata*, and *Rhodosporidium babjevae*. Lipases are implemented in vast commercial applications, including detergents and cosmetics additives, fine chemical production, medical application, paper pitching, leather de-fating, wastewater treatment, and biodiesel production. The application of lipase in biodiesel production, as an ecofriendly alternative for traditional fuel, intensifies the research in diminishing the production cost and enhancing the enzyme efficiency.

Proteases

Proteases play an important role in fungal physiology to digest extracellular large peptides and also in defense mechanisms against attaching pathogens. Based upon the amino acid in the enzyme active site, proteases could be categorized into different types, including serine, asparagine, cysteine, aspartic, and metalloproteases. Serine and metalloprotease are the most studied types among all proteases and are usually produced from microbial origins. Filamentous fungi, especially that of *Aspergillus* sp. are characterized by their high capacity for protease production. Other fungal genera also reported for their potency regarding proteases production, including *Penicillium* sp., *Fusarium* sp., and *Pichia farinosa*.

Cellulases

Cellulose, hemicellulose, and lignin are the main components of most agricultural wastes. Most fungi have the complete enzymatic system (Endoglucanases, Cellobiohydrolases, β-glucosidases, and Xylanases) to degrade this complex cellulosic material for nutrition. *Trichoderma reesei* is widely applied for the commercial production of cellulases, other fungi also represent potent cellulase producers, including *Aspergillus niger*, *Saccharomyces cerevisiae*, and *Aspergillus brasiliensis*. Xylan, a complex polysaccharide, is also a major component of hemicellulose; hence, xylanases play an important role in the efficient hydrolysis of plant cellulolytic material. Regarding the diverse and complex structure of Xylan, its hydrolysis required a group of synergistically working enzymes (xylanolytic system) for complete degradation. Filamentous fungi are characterized by the required xylanolytic system for complete xylan degradation, especially that of *Trichoderma reesei*. *Aspergillus oryzae*, and *Aspergillus flavus*.

Detection of amylase produced by fungi

Procedures

1- Preparation of culture media for amylase production and sterilization

2- Cultivation of fungi

3- Incubation of cultures under suitable temperature and pH

3- At the end of the incubation period, filtration of cultures and assay for the enzyme activity.

4- Illustrate the data and write a comment.

Table 6:……..……..…

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Fungi	Amylase activity

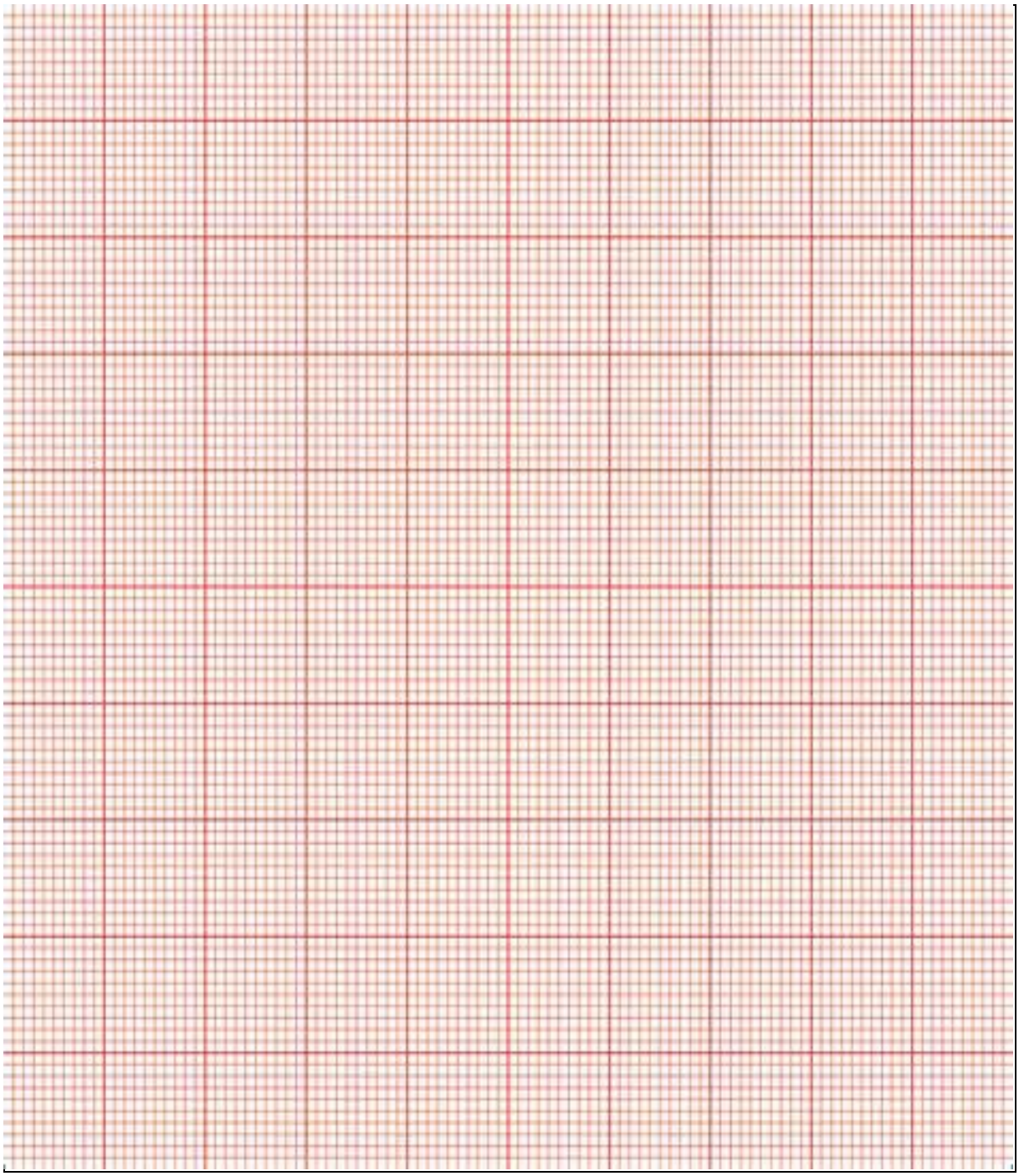

Figure 6: ……..

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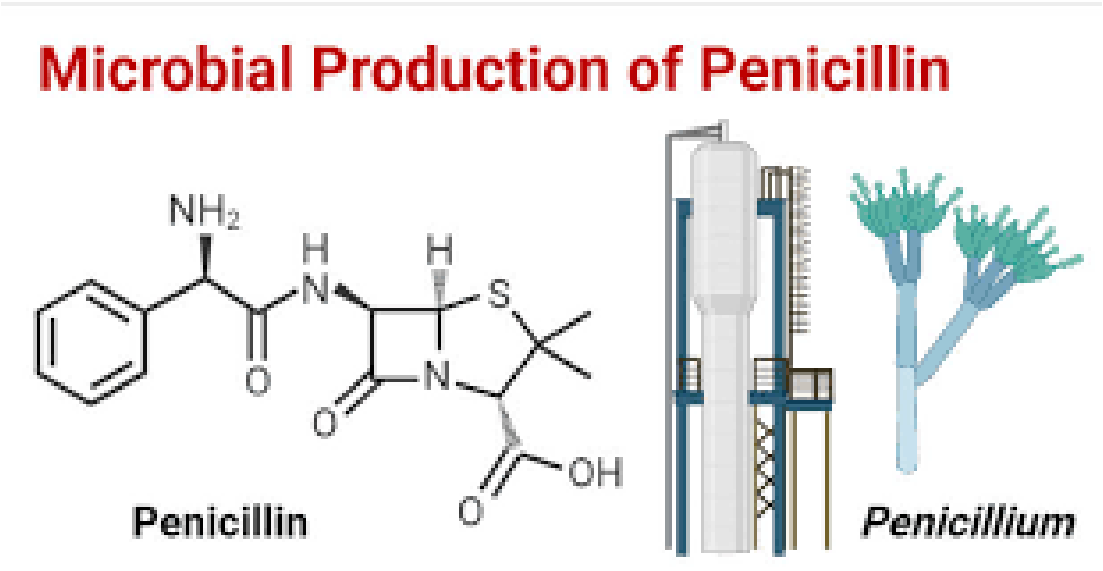
Experiment 7

Antifungal agents

A wide range of antifungal agents are used in combating biodeterioration and in preventing or treating fungal diseases of plants. In these contexts, they are commonly referred to as **fungicides**. Others are used for treating disease in animals and man, and are simply referred to as **antifungal agents.** Antimicrobial agents produced by means of a microbial fermentation, called **antibiotics**, by the plant on which the mold is growing, or added as biocides during crop management, are other factors interacting with the growth and metabolism of a mold. Antifungal agents differ widely in their chemical nature and in their properties and mode of action (Carlile and Watkinson, 1996). The effect of pesticides is interesting as they are largely used to control several diseases in plants. The correct use of fungicides to diminish fungal mycoflora could lead to a diminution in the amount of mycotoxins produced. But certain number of studies showed that the use of sub-lethal concentration could favour the production of the toxins (Moss and Frank, 1987). It is also possible that the pesticide decreases the synthesis of the mycotoxins without affecting the fungal growth (Draughton and Ayres, 1978, 1982).



Production of Penicillin by *Penicillium chrysogenum*

Procedures

1- Cultivation of fungi on a suitable media for penicillin production.

2- Incubation of cultures at suitable conditions (Temperature, pH, etc.).

3- At the end of the incubation period, filtration of cultures.

4- Extraction of penicillin using a suitable solvent.

5- Collection of solvent with the antibiotic.

6- Concentration of solvent by rotary evaporation.

7- Collection of solvent and dissolve of penicillin in methanol.

8- Analysis of penicillin for detection and concentration by TLC, HPLC, etc.

9- Illustrate the data and write a comment.

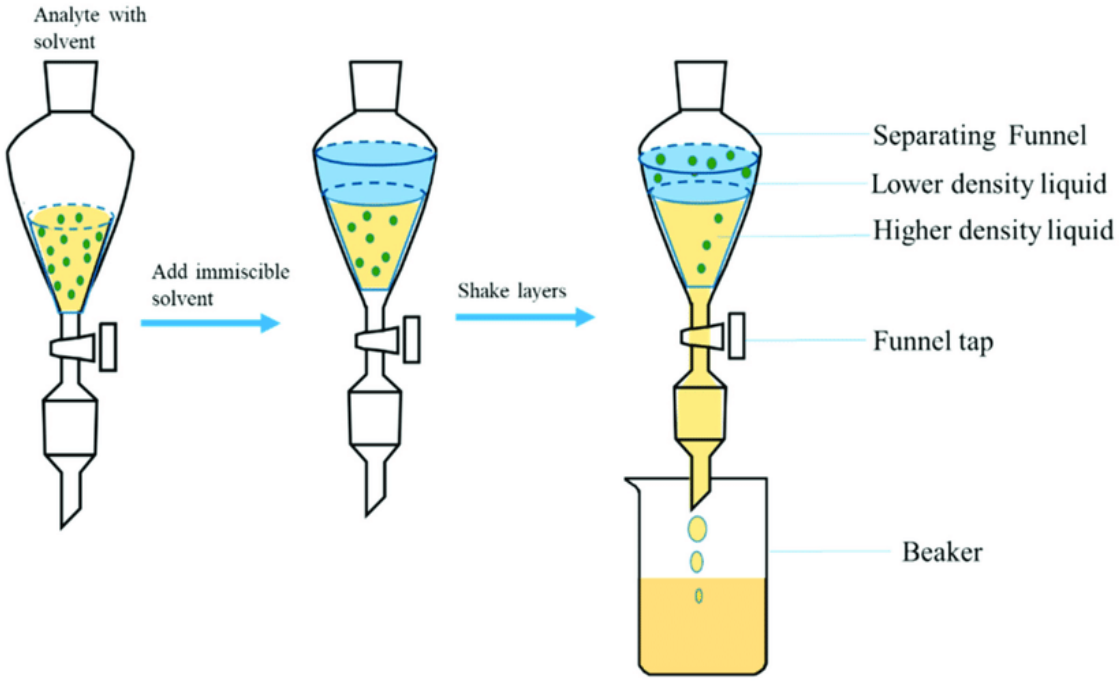


Table 7:……..……..…

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Penicillium strains	Penicillin activity
1	
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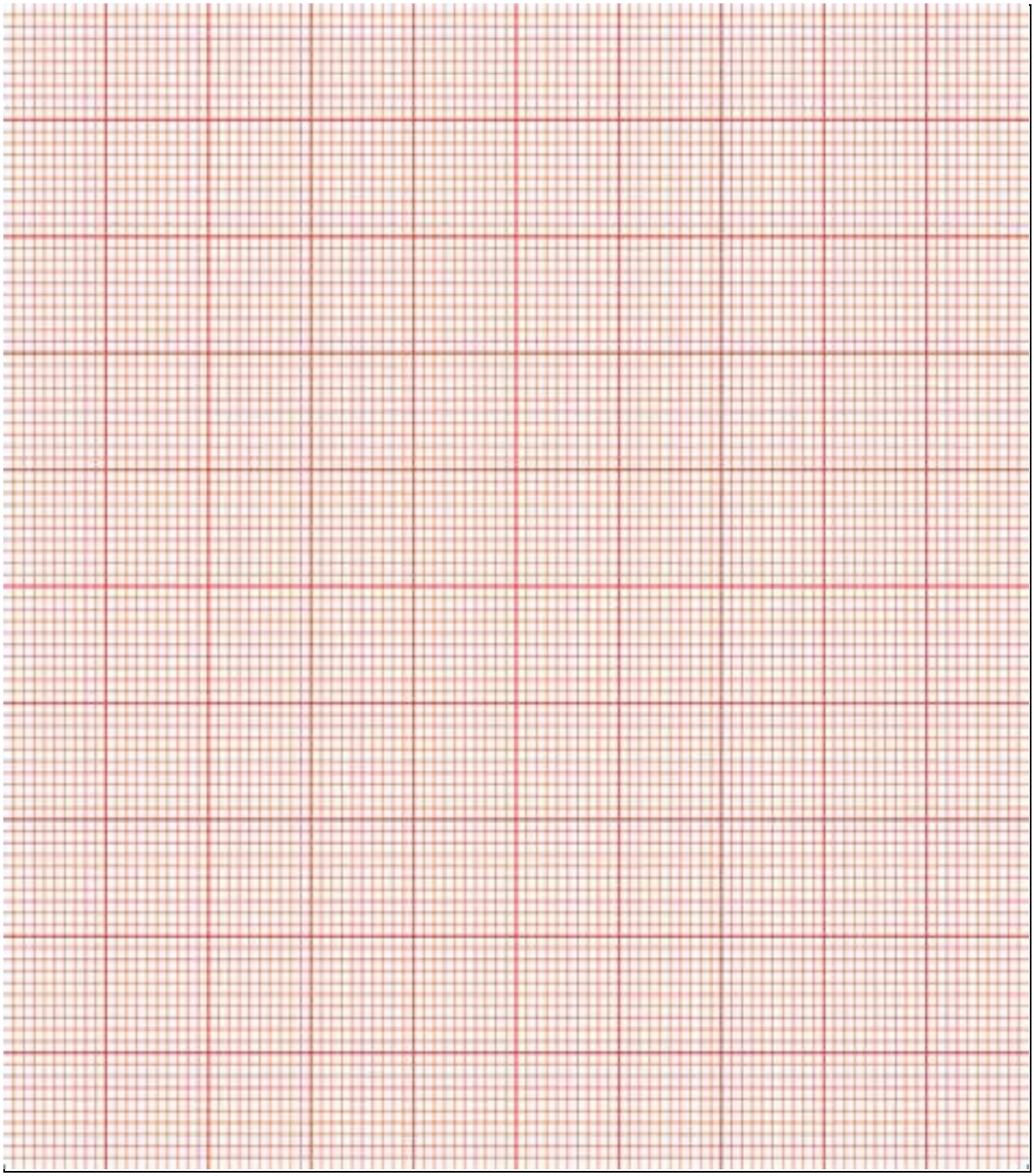

Figure 7: ……..

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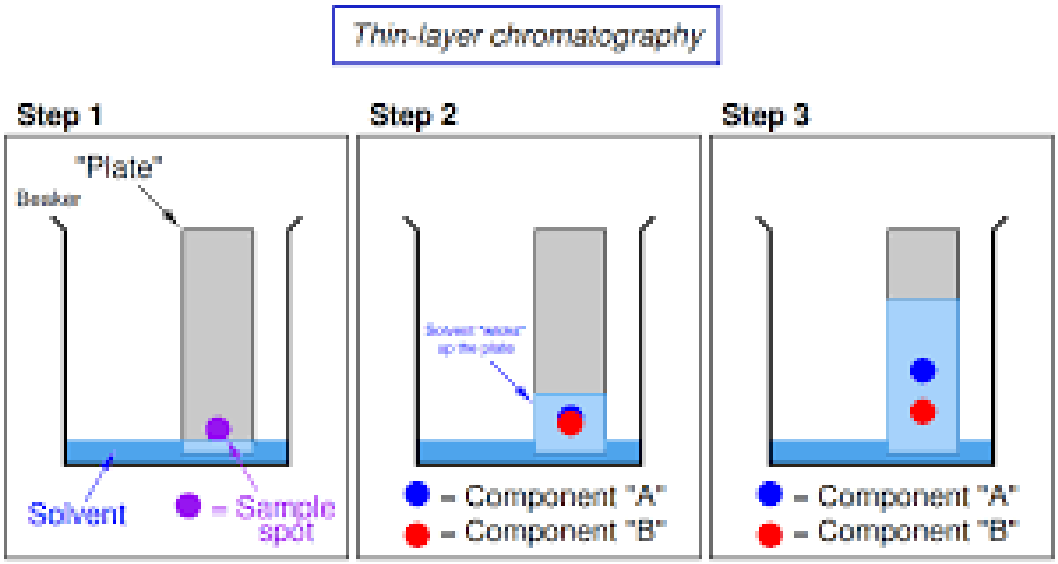
Methods for the analysis of antibiotics

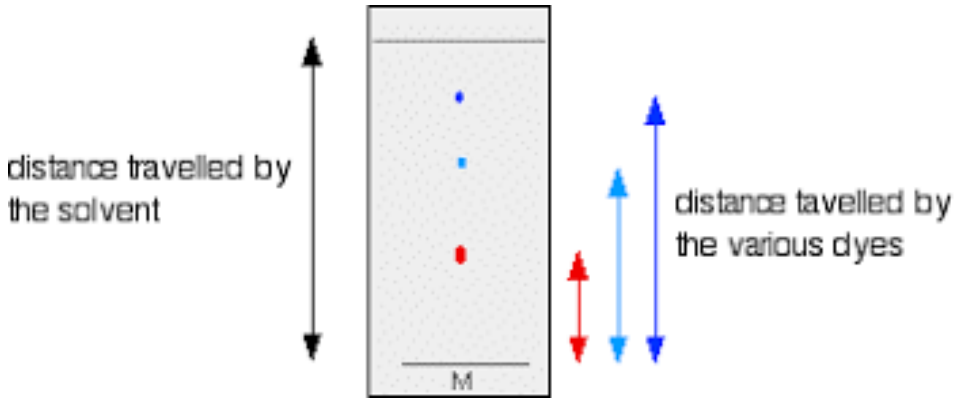
The different stages involved in the analytical process of antibiotics detection are sampling, extraction, clean-up, separation, detection and confirmation. Although many interfering compounds may be partially removed during the extraction sequence, further clean-up of the extract is normally necessary. The traditional clean-up systems generally involved either solvent portioning and/or open column chromatography on silica adsorbent. The development of solid phase extraction (SPE) cartridges containing packing with various surface chemistries allowed more rapid and efficient clean-up process. However, the introduction of the immunoaffinity columns (IAC) in which specific antibodies are bound to a solid matrix, has allowed an even more specific clean-up process. Classical analytical separation methods for antibiotics include TLC, HPLC, gas chromatography (GC) and MS. Mass spectrometry offers the ideal confirmatory technique via the detection of molecular ions at specific chromatographic retention times and via the generation of a compound specific fragmentation pattern.

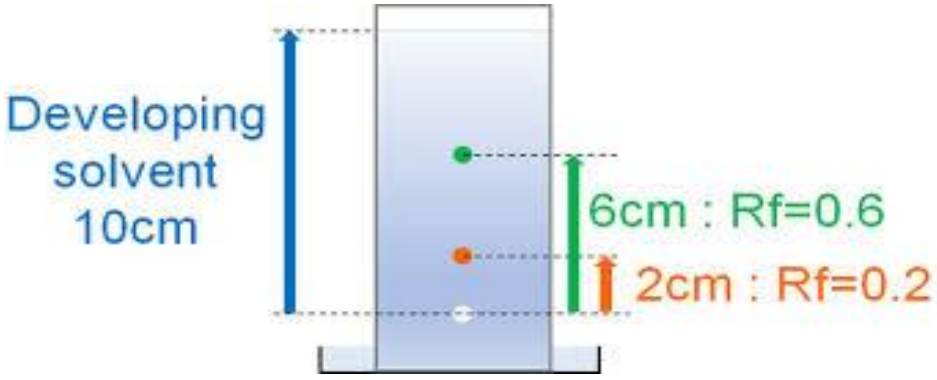
Thin Layer Chromatography (TLC) analysis

Thin layer chromatography, or TLC, is a method for analyzing mixtures by separating the compounds in the mixture. TLC can be used to determine the number of components in a mixture, the identity of compounds, and the purity of a compound. By observing the appearance of a product or the disappearance of a reactant, it can also be used to monitor the progress of a reaction. TLC is a sensitive technique - microgram (0.000001 g) quantities can be analyzed by TLC. TLC consists of three steps: spotting, development, and visualization. First the sample to be analyzed is dissolved in a volatile (easily evaporated) solvent to produce a very dilute (about 1%) solution. Spotting consists of using a micro pipet to transfer a small amount of the dilute solution to one end of a TLC plate, in this case a thin layer of powdered silica gel that has been coated onto a plastic or glass sheet. The spotting solvent quickly evaporates and leaves behind a small spot of

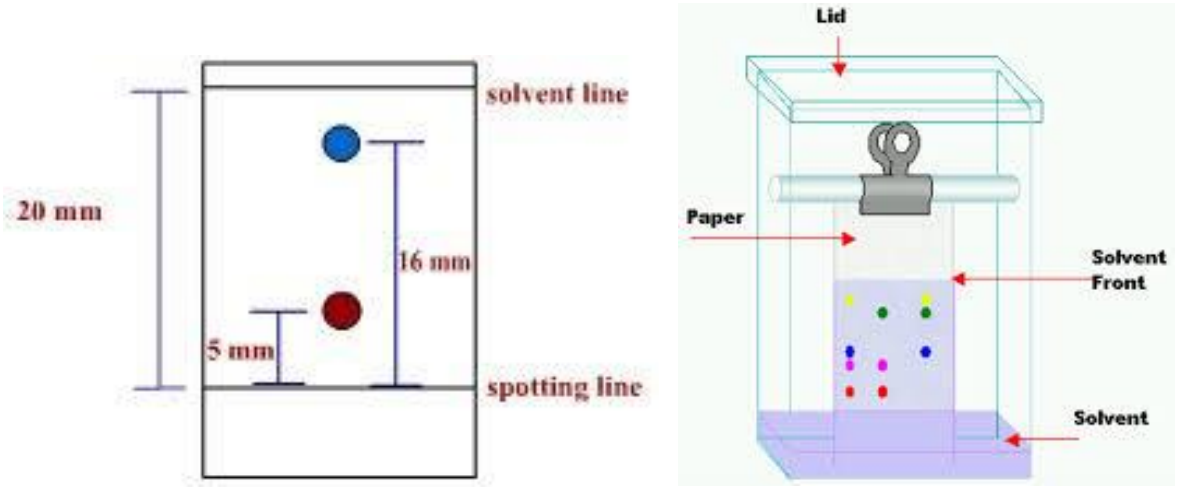
the material. Development consists of placing the bottom of the TLC plate into a shallow pool of a development solvent, which then travels up the plate by capillary action. As the solvent travels up the plate, it moves over the original spot. A competition is set up between the silica gel plate and the development solvent for the spotted material. The very polar silica gel tries to hold the spot in its original place and the solvent tries to move the spot along with it as it travels up the plate. The outcome depends upon a balance among three polarities - that of the plate, the development solvent and the spot material. If the development solvent is polar enough, the spot will move some distance from its original location. Different components in the original spot, having different polarities, will move different distances from the original spot location and show up as separate spots. When the solvent has traveled almost to the top of the plate, the plate is removed, the solvent front marked with a pencil, and the solvent allowed to evaporate. Visualization of colored compounds is simple–the spots can be directly observed after development. Because most compounds are colorless however, a visualization method is needed. The silica gel on the TLC plate is impregnated with a fluorescent material that glows under ultraviolet (UV) light. A spot will interfere with the fluorescence and appear as a dark spot on a glowing background. While under the UV light, the spots can be outlined with a pencil to mark their locations. A second method of visualization is accomplished by placing the plate into iodine vapors for a few minutes. Most organic compounds will form a dark-colored complex with iodine. It is good practice to use at least two visualization techniques in case a compound does not show up with one particular method. The Rf value is used to quantify the movement of the materials along the plate. Rf is equal to the distance traveled by the substance divided by the distance traveled by the solvent. Its value is always between zero and one.







$$Rf = \frac{\text{distance moved by the compound}}{\text{distance moved by the solvent}}$$

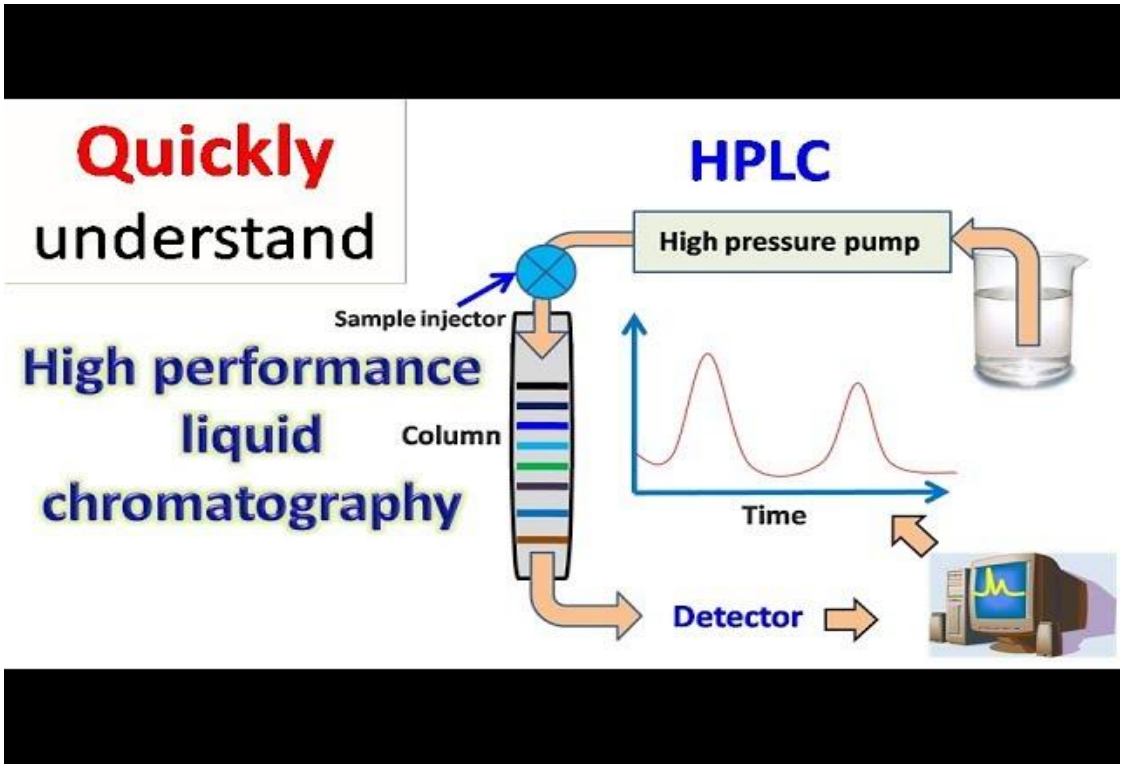


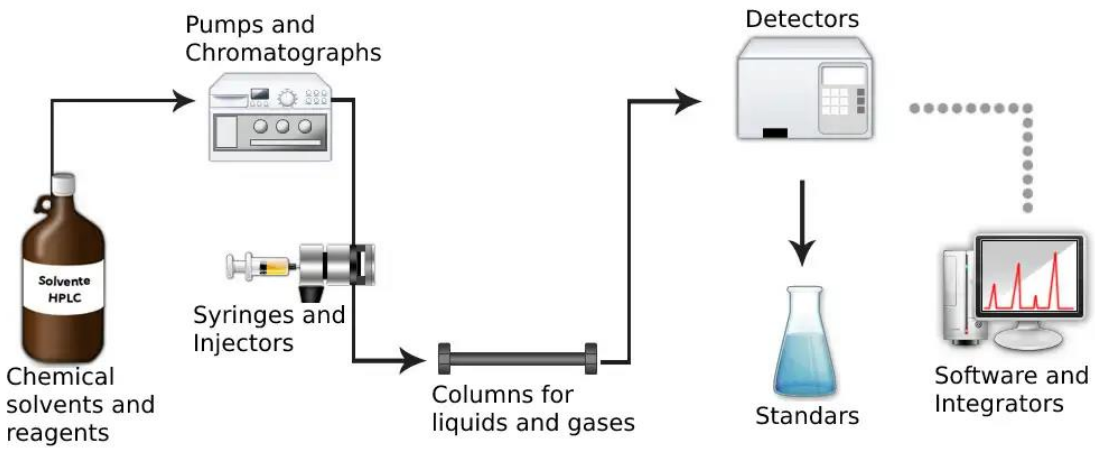
High-performance liquid chromatography (HPLC) analysis

High-performance liquid chromatography (HPLC), formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (*e.g.*, silica, polymers, etc.), 2–50 μm in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (*e.g.*, water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and

adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.

The schematic of an HPLC instrument typically includes a degasser, sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in an HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/V is, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.





High-performance liquid chromatography (HPLC)

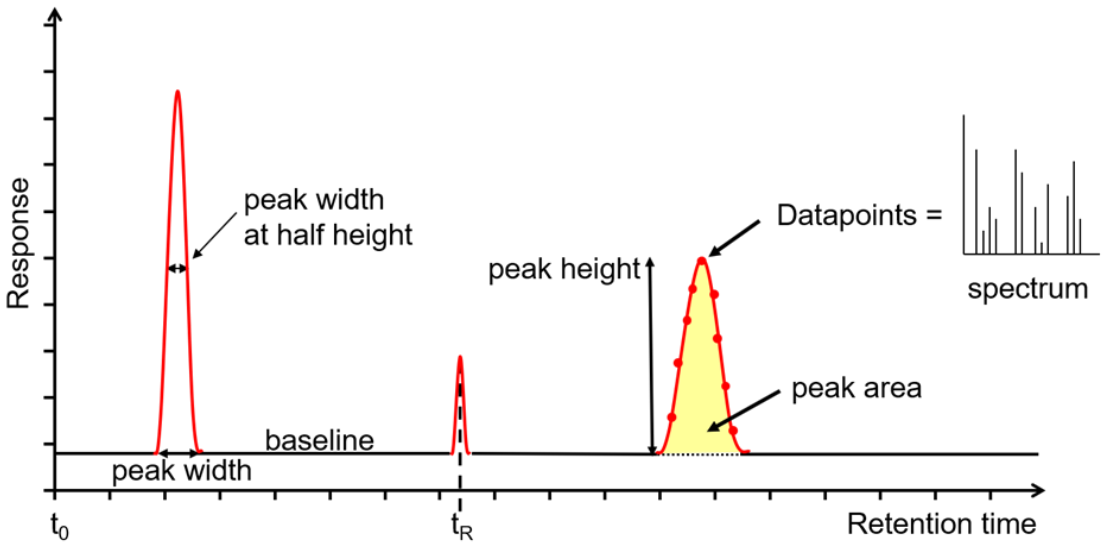
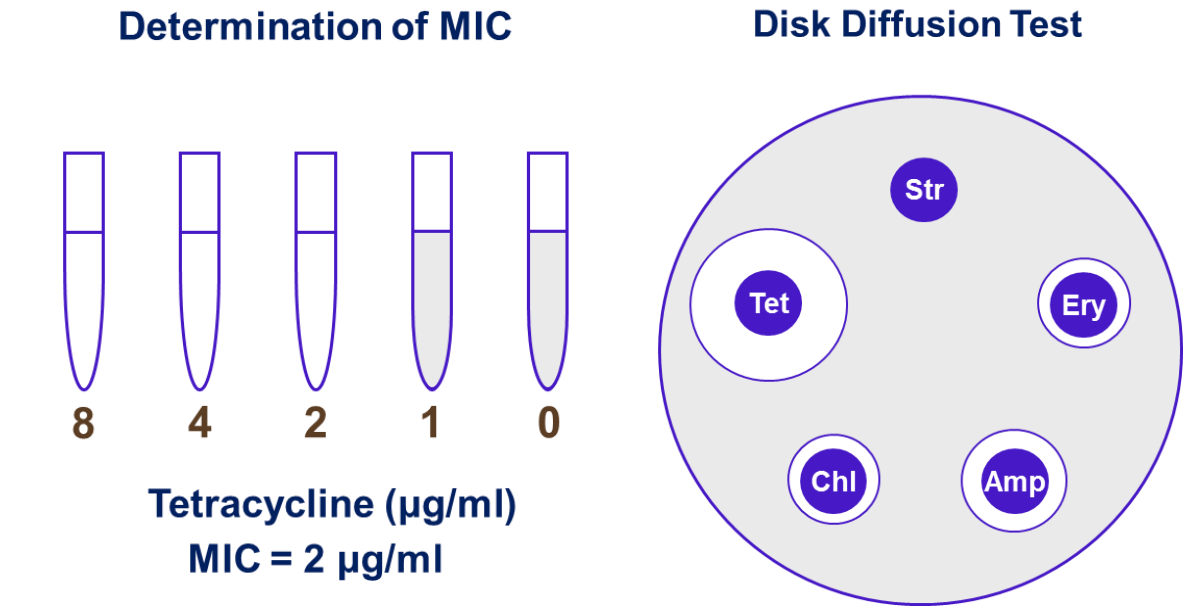
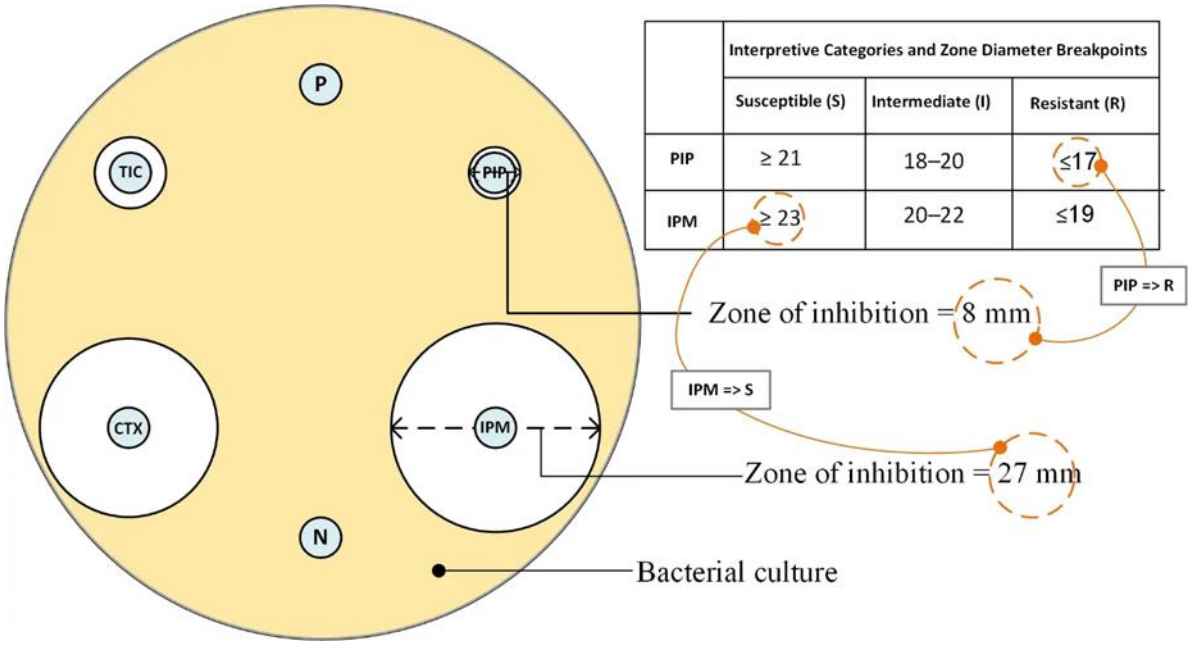


Chart of HPLC analysis

Susceptibility testing of antibiotics (Minimum inhibitory concentration, MIC).





	Interpretive Categories and Zone Diameter Breakpoints		
	Susceptible (S)	Intermediate (I)	Resistant (R)
PIP	≥ 21	18–20	≤17
IPM	≥ 23	20–22	≤19

Procedures

1- Preparation of suitable medium for antibiotic testing.

2- Cultivation of bacteria.

3- Placed of discs on the Petri-dish and added different antibiotics or different antibiotic concentrations.

4- Incubation of dishes for 24 or 48 hours.

5- Measure the inhibition zones around the discs and calculate the averages.

6- Determine the activity and MIC of the antibiotics.

7- Illustrate the data and write a comment.

Table 8:……..……..…

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Antibiotics	Inhibition zone (mm)	MIC

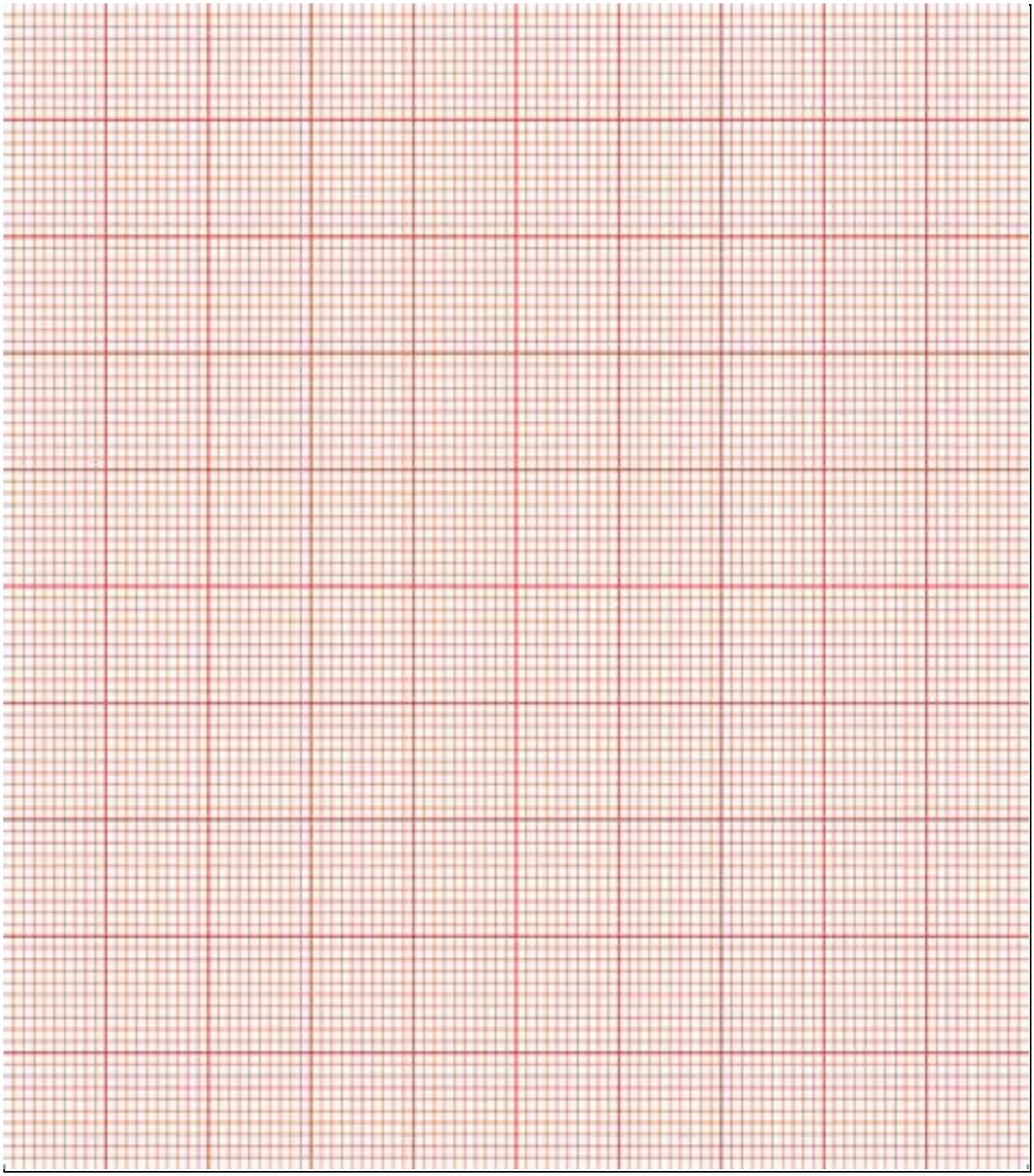

Figure 8: ……..

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Practical part

Fungi

4th year Chemistry &Botany

Dr. Amany Atta El-Shahir

Safety Procedures for the Microbiology Laboratory

General Laboratory Safety Practices and Procedures

1. If you are **taking immune-suppressants, are pregnant,** or have a known medical condition that would prevent full participation in the laboratory, please contact the course instructor before the first day of lab.
2. Read and understand each laboratory exercise **before** you come to class.
3. Do not eat, drink, smoke, or chew pens in the laboratory.
4. You must wear close-toed shoes while in the laboratory and long pants.
5. No hats of any kind will be allowed in lab, unless allowed by University policy and cleared with the instructor.
6. Long hair should be pulled back to keep it away from bacterial cultures, bacticinerator or open flames.
7. Follow precautionary statements given in each exercise.
8. Personal electronic devices will be turned off and stored while in this laboratory. *The unauthorized use of any electronic device (phone, tablet, computer) in lab will result in a loss of course points.
9. Know where specific safety equipment is located in the laboratory, such as the fire extinguisher, safety shower, and the eyewash station.
10. Recognize the international symbol for biohazards, and know where and how to dispose of all waste materials, particularly biohazard waste. Note that all biohazard waste must be sterilized by autoclave before it can be included in the waste stream.

Figure 1: Biohazard Symbol

11. Keep everything other than the cultures and tools you need OFF the lab bench. Only necessary work material should be at or on the laboratory bench. Coats, backpacks, and other personal belongings will not be allowed on the laboratory bench top. Store them in a place designated by your instructor. This is to prevent cluttering of the workspace and to avoid exposing them to permanent stains, caustic chemicals, and microorganisms used in the exercises.
12. Leave all laboratory facilities and equipment in good order at the end of each class. Before leaving the laboratory, check to make sure the bacticinerator heat sterilizer is turned off.
13. Never, under any circumstances, remove equipment, media, or microbial cultures from the laboratory.
14. No pets are allowed in the laboratory.

Microbiology Specific Laboratory Safety Practices

During the course of the semester in the laboratory you will be taught the methods used in the proper handling of microorganisms. Although you will not be working with any that are human pathogens, exercise caution in handling all material coming in contact with live microbial cultures. All cultures should be handled with respect and proper aseptic technique *as if they were potential pathogens.* This is called **"universal precaution"**. Specific instructions that should be followed:

1. Remember that all bacteria are potential pathogens that may cause harm under unexpected or unusual circumstances. If you as a student have a compromised immune system or a recent extended illness, you should share those personal circumstances with your lab instructor.
2. Wear gloves when working with cultures, and when your work is completed, dispose of the gloves in the biohazard garbage. Lab coats, safety glasses or goggles are also required. These will be stored in the laboratory each week in a ziplock bag.
3. Disinfect your work area both BEFORE and AFTER working with bacterial cultures.
4. Cultures of live microorganisms and any material coming in contact with live cultures must be properly sterilized after use in the laboratory. Your instructor will inform you of specific procedures. Follow the general rules outlined below.

   a. Glassware such as test tubes, bottles, and flasks may be reused and washed after sterilization. These are normally placed on a cart at the front of the laboratory after you have finished an experiment or exercise. BE SURE TO REMOVE LABELS before placing any glassware on the cart. Your instructor will sterilize and then wash these items.

   b. Some materials, such as plastic petri dishes, plastic pipettes, microscope slides, and swabs, are considered disposable. These are used once and if they become contaminated by contact with live microorganisms are sterilized and discarded. All of these disposable contaminated materials should be placed in the designated waste container containing a BIOHAZARD autoclave bag.

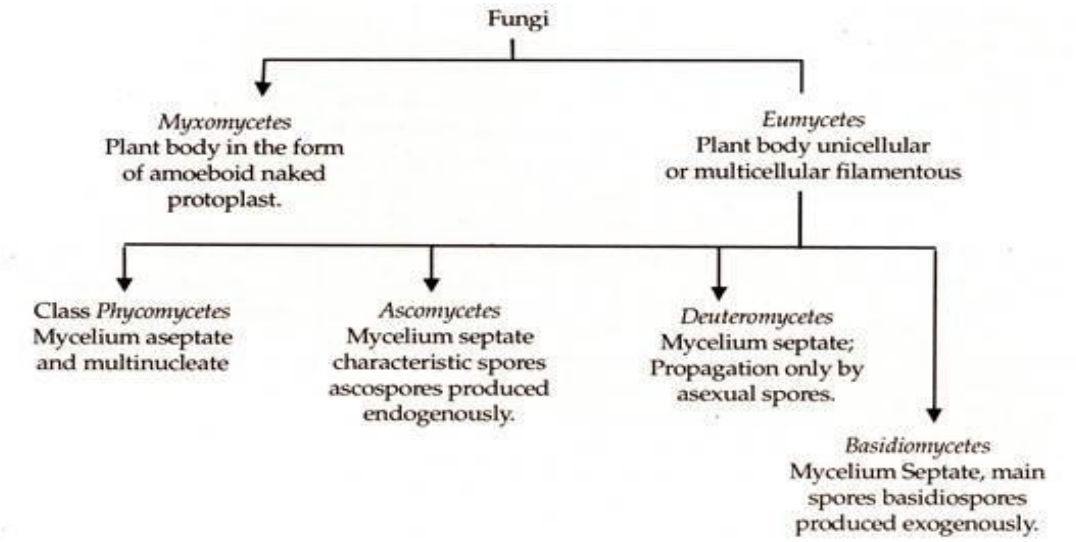
5. Never place contaminated pipette tips (or pipettes), inoculating loop, or any other contaminated material on the bench top. Sterilize loops before and after each use. Place contaminated pipette tips in the orange biohazard buckets on your bench. Place all other contaminated materials in their designated waste containers. Do not place or put anything containing live microorganisms in the sink.
6. Aerosols should be avoided by the use of proper technique for sterilizing the inoculating loops and by performing any mixing of cultures and reagents in such a way as to avoid splashing.
7. Cultures or reagents should always be transferred with an automatic pipettor that will be provided. In no case should one employ mouth pipetting.

8. Always keep cultures capped and in proper storage racks when not being used during an exercise.
9. In the event of an accidental spill involving a bacterial culture, completely saturate the spill area with disinfectant, then cover with paper towels and allow the spill to sit for 10 minutes. Then carefully remove the saturated paper towels, dispose of them in the biohazard waste, and clean the area again with disinfectant. Notify your instructor about the spill. If the chemical is marked "danger" or "caustic" you should notify the instructor who will handle this type of spill.
10. Immediately report all accidents such as spills, cuts, burns, or other injuries to the instructor
11. Make sure that lab benches are completely cleared (everything either thrown away or returned to storage area) before you leave the lab.
12. Clothing worn in the microbiology laboratory should be washed before being subsequently worn in a facility such as a hospital, clinic or nursing home, or in an area of public food preparation.
13. In the event of a fire alarm, follow the directions of your instructor, and meet at the place designated by your instructor.

Classifications

Alexopoulos (1956) places all fungi in the division Mycota. The division Mycota is divided into two subdivisions (1) Myxomycotina (2) Eumycotina (true fungi). Myxomycotina has only one class – Myxomycetes.

Eumycotina has the four classes as shown in the figure.



K. Mycophyta

D. Myxomycophyta

C. Myxomycetes

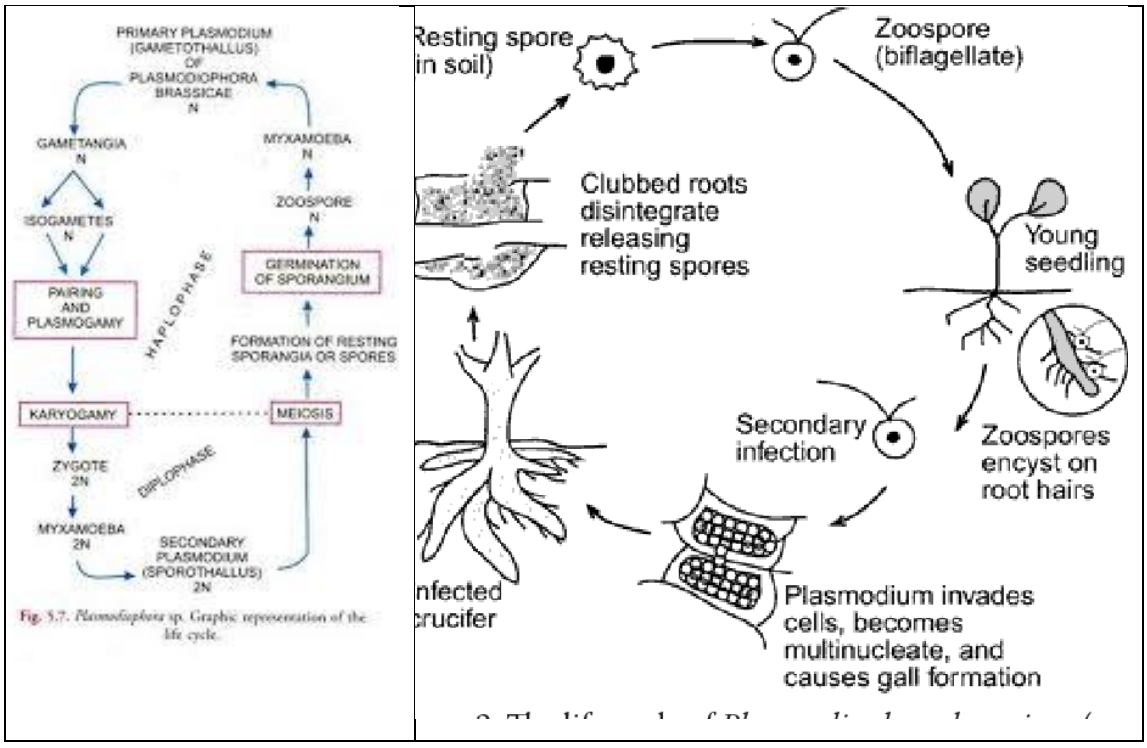
O: *Plasmodiophorales*

F: *Plasmodiophoraceae*

Ex. *Plasmodiophora brassicae*

Please check images in the following link

https://www.shutterstock.com/search/plasmodiophora-brassicae

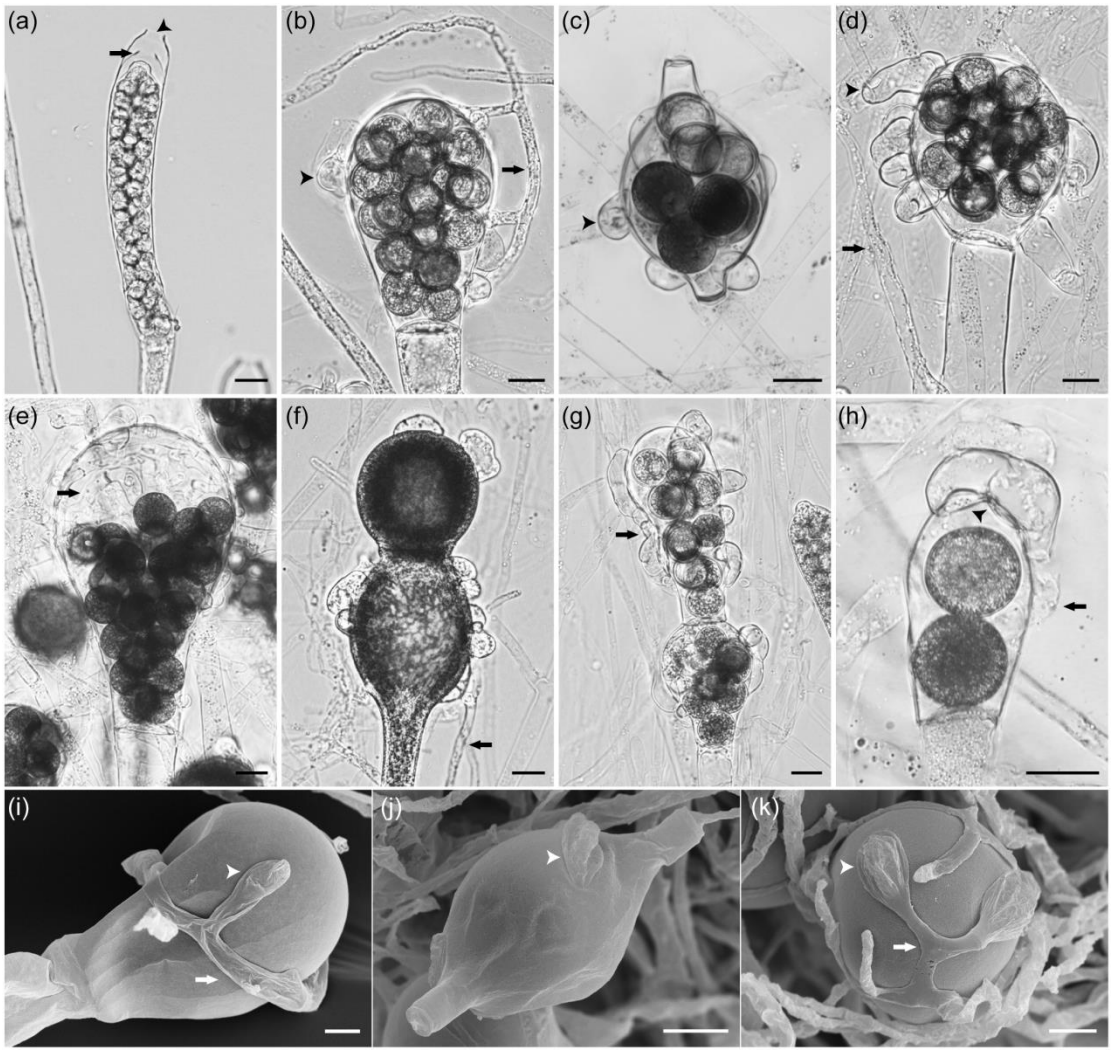


C: Phycomycetes

O: Saprolegniales

F: Saprolegniaceae

Ex.: *Saprolegnia* sp.

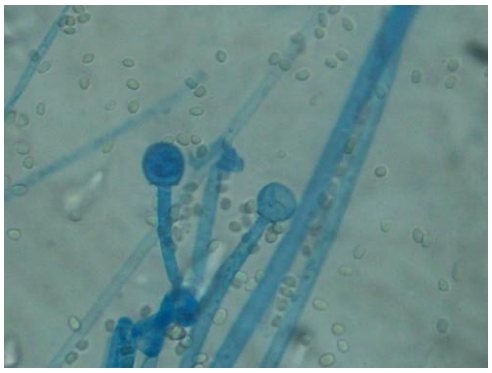


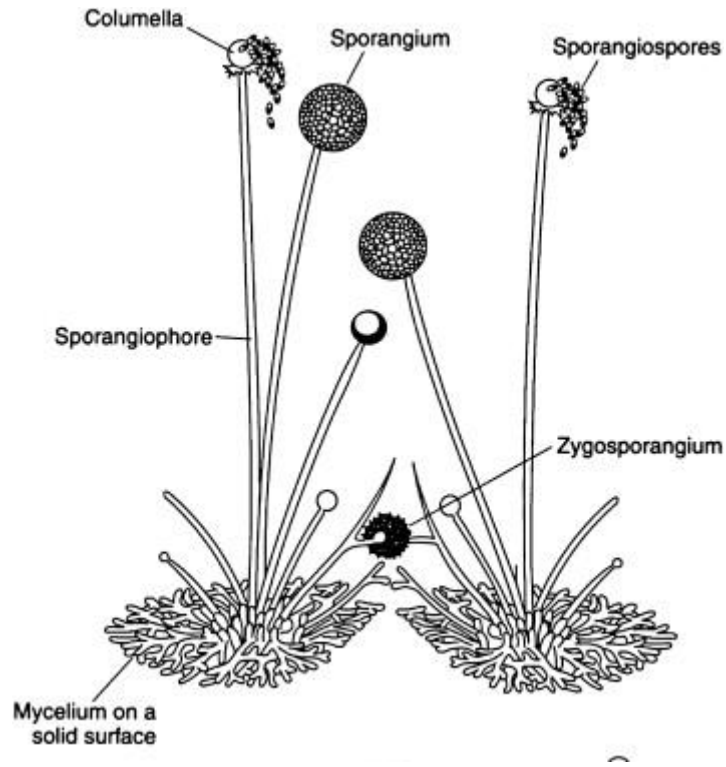
C: *Phycomycetes*

O: *Mucorales*

F: *Mucoraceae*

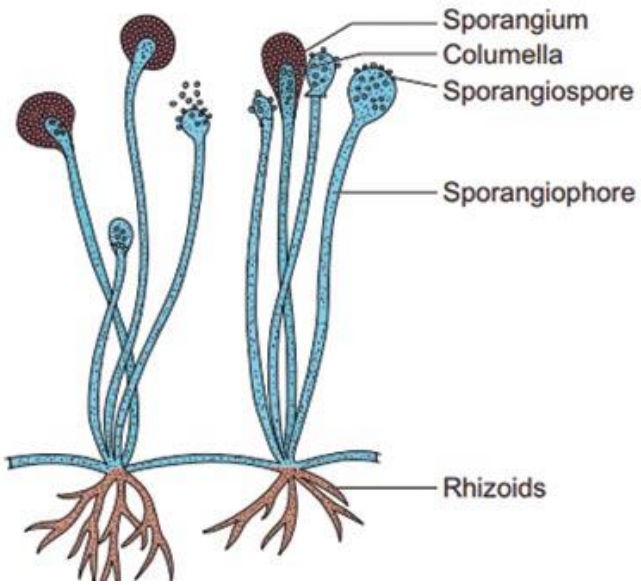
Ex.1. *Mucor racemosus*



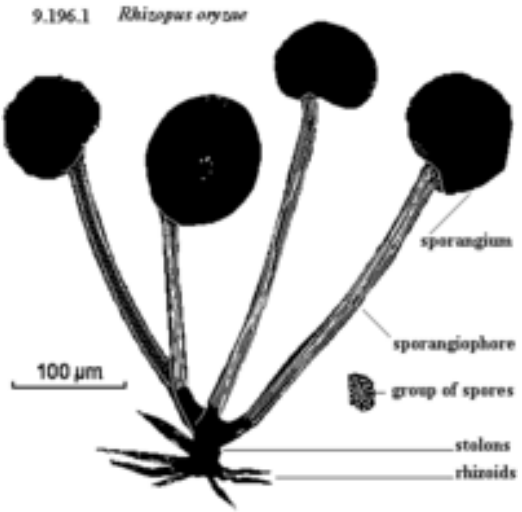


Ex. 2: *Rhizopus stolonifer*

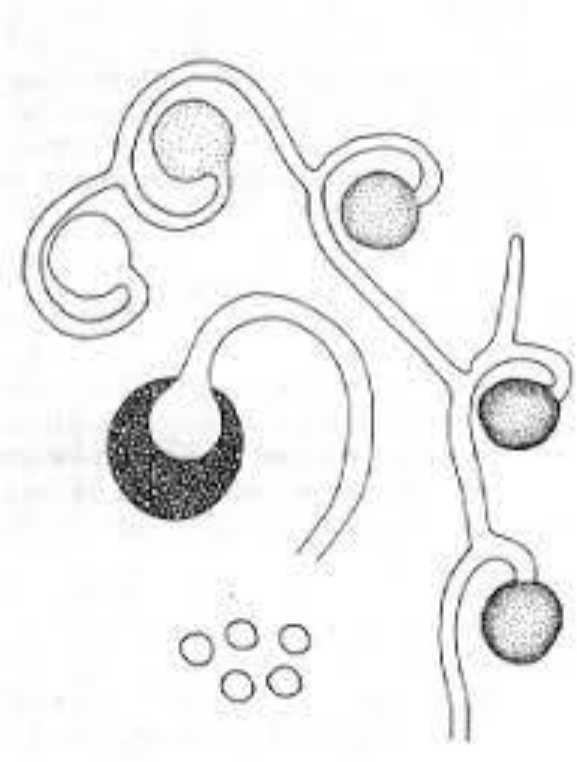
Rhizopus is **a genus of saprophytic and parasitic fungi**. They are found in moist or damp places. They are found on organic substances like vegetables, fruits, bread, jellies, etc. The vegetative structure is made up of coenocytic (multinucleated) and branched hyphae.

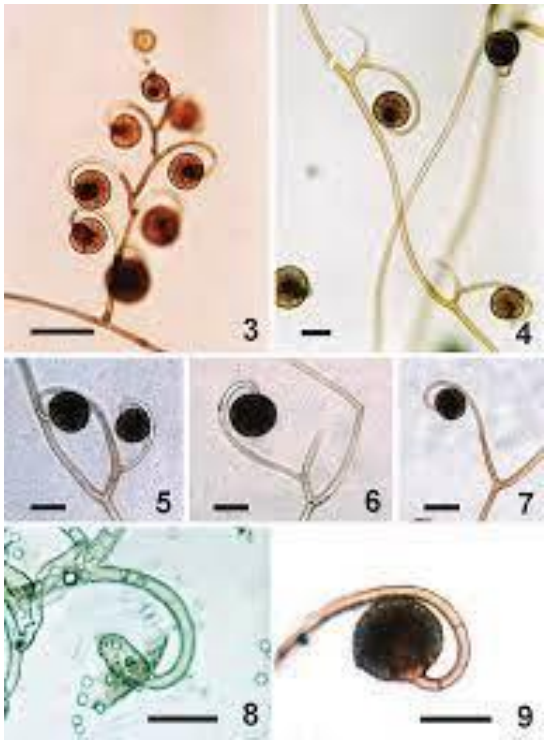


R. oryzae



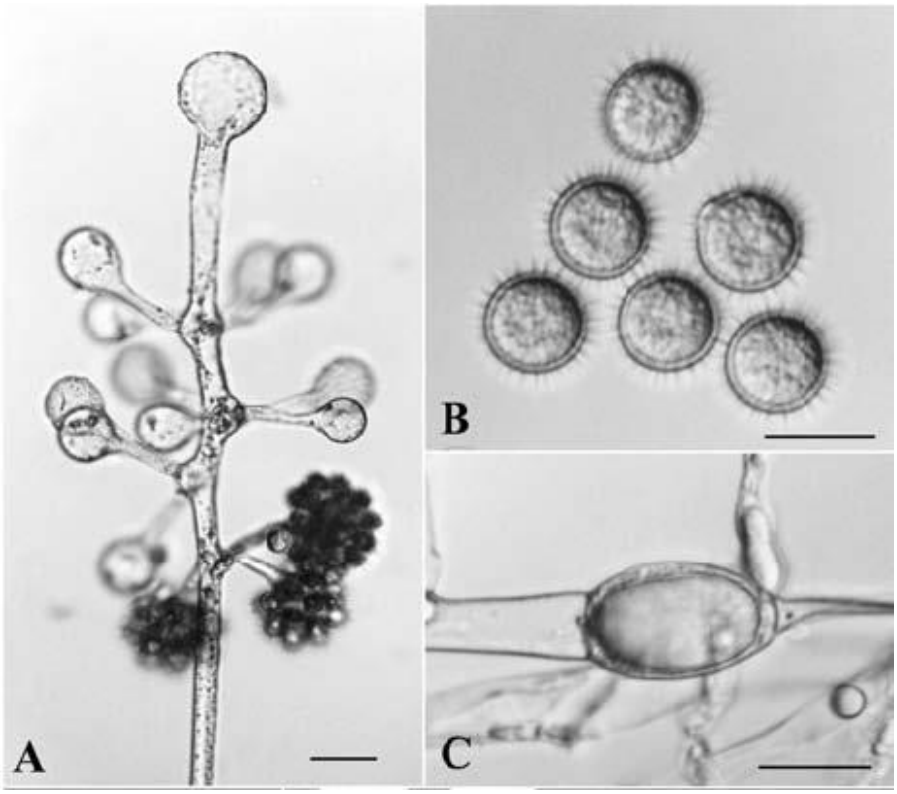
Ex. 3 *Circinella* sp.





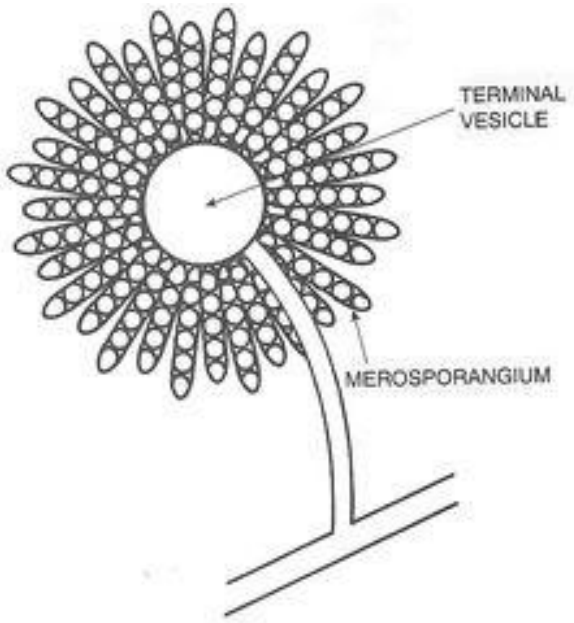
Family 2: *Choanephoraceae*

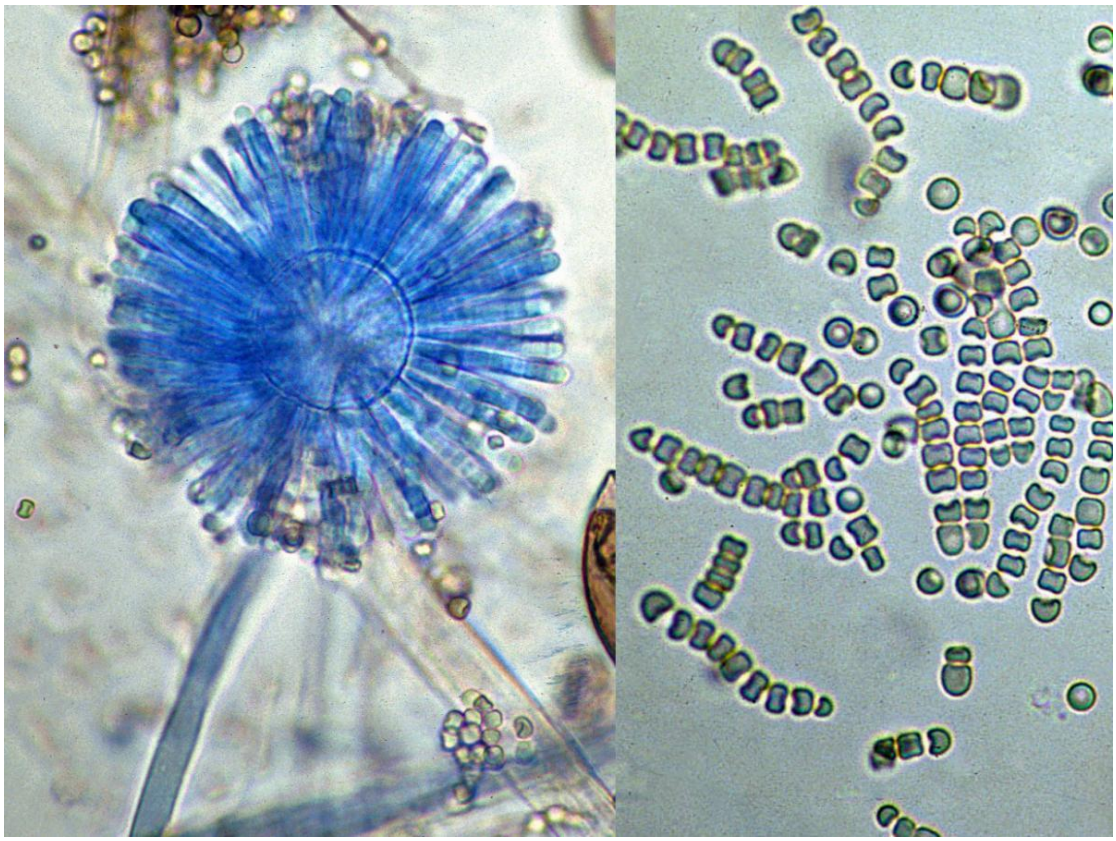
Ex. *Cunninghamella echinulata*



Family 3 : *Cephalidaceae*

Ex. *Syncephalastrum* sp.





Class 2: *Ascomtcetes*

Subclass: *Euascomycetes*

Series 1: *Plectomycetes*

Order 1: *Aspergillalaes*

Family : *Aspergillaceae*

Ex.1 *Aspergillus*

Different *Aspergillus* sp.

1	*Aspergillus clavatus*
2	*Aspergillus chevalieri*
3	*Aspergillus fumigatus*
4	*Aspergillus candidus*
5	*Aspergillus flavus*
6	*Aspergillus ochraceus*
7	*Aspergillus niger*
8	*Aspergillus versicolor*
9	*Aspergillus nidulans*
10	*Aspergillus ustus*
11	*Aspergillus flavipes*
12	*Aspergillus terreus*

General characteristics

1- Colony colour
2- Colony reverse
3- Sterigmata: Biserriate – uniserriate
4- Conidia: globose – subglobose –elliptical – ovate – rough – smooth – hyaline – pigment.
5- Vesicle : globose – subglobose – clavate.
6- Conidial head: radiate – columnar – clavate.
7- Conidiophore: long – short – branched – unbranched – smooth – rough – hyaline – pigment – straight – sinuate.
8- Ascospre
9- Hull cell
10- Sclerotia

Ex. 2: Penicillium

General characteristics

1- Colony colour
2- Colony reverse
3- Metulae: Present – Absent
4- Penicillin: Monoverticillata – biveticillata – symmetrica – asymmetrica – divaricate – nondivaricata (velutina – lanata – fasiculata).
5- Conidia: globose – subglobose –elliptical – ovate – rough – smooth – hyaline – pigment.
6- Conidiophore: long – short – branched – unbranched – smooth – rough – hyaline – pigment – straight – sinuate.
7- Ascospre
8- Hull cell
9- Sclerotia

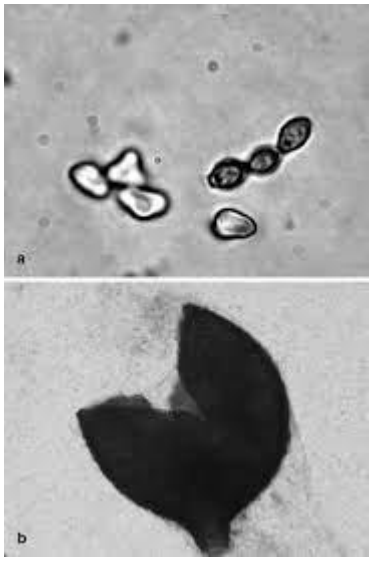
Different *Penicillium* sp.

1	*Penicillium corylophilum*
2	*Penicillium duclauxi*
3	*Penicillium funiculosum*
4	*Penicillium chrysogenum*
5	*Penicillium steckii*
6	*Penicillium waksmani*
7	*Penicillium purpurogenum*
8	*Penicillium corylophilum*
9	*Penicillium duclauxi*
10	*Penicillium funiculosum*
11	*Penicillium chrysogenum*
12	*Penicillium steckii*
13	*Penicillium waksmani*
14	*Penicillium purpurogenum*

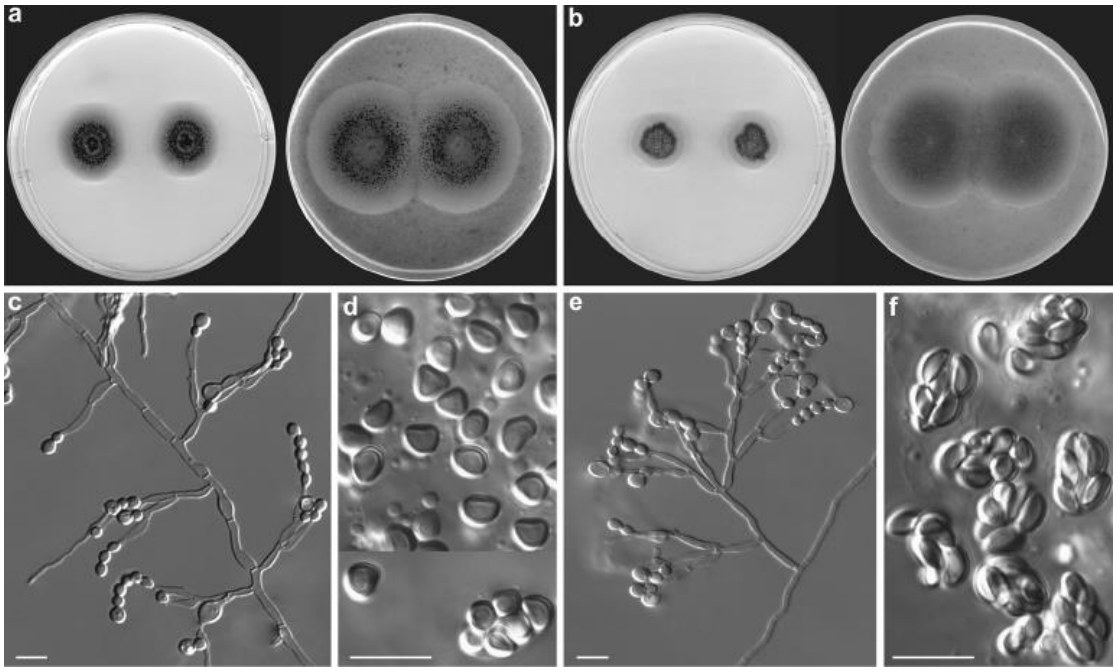
Order 2: Microascales

Family: Microascaceae

Ex. *Microascus trigonosporus* **(الجراثيم الزقية مثلثة الشكل)**



M. cinereus **(الجراثيم الزقية كلوية الشكل)**

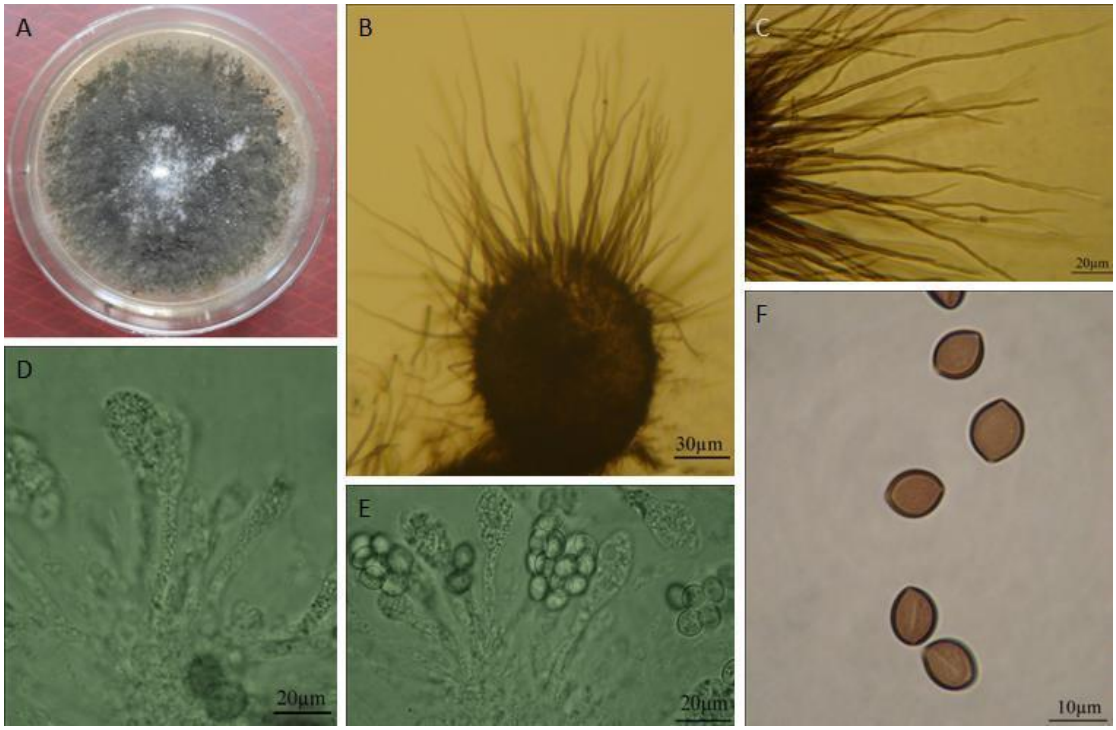


Series 2: *Pyrenomycetes*

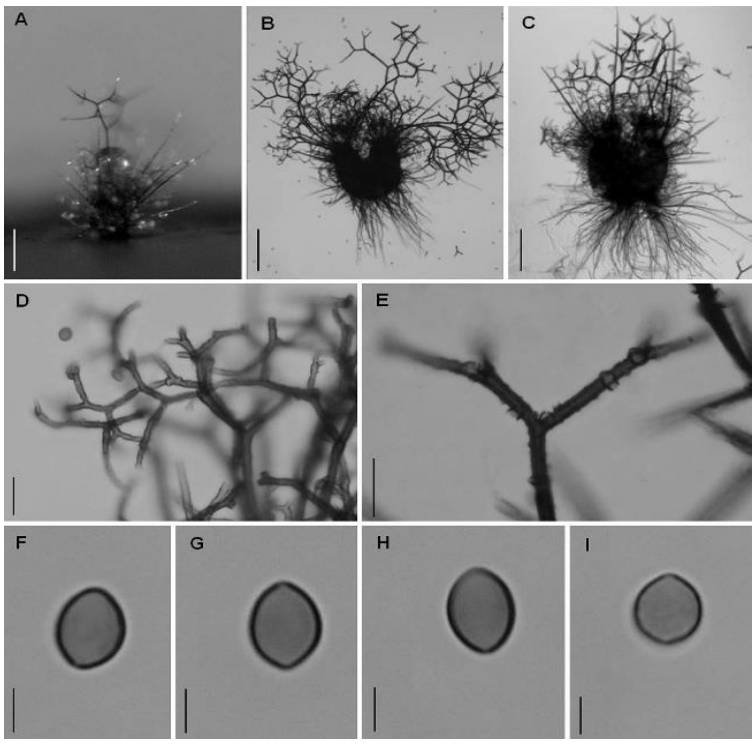
Order: *Sphaeriales*

Family: *Chaetomiaceae*

Ex.: Cheatomoium globosum



C. funicola



C. dreyfussi

C. uniporum

C. atrobrunneum

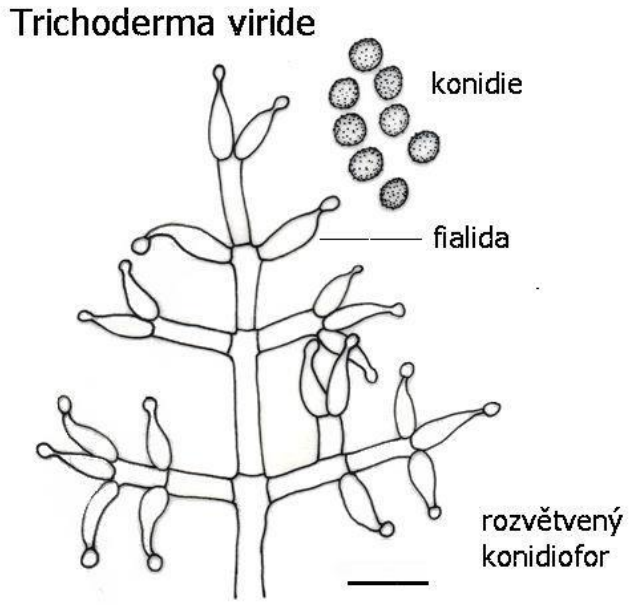
Class 3 : *Deuteromycetes*

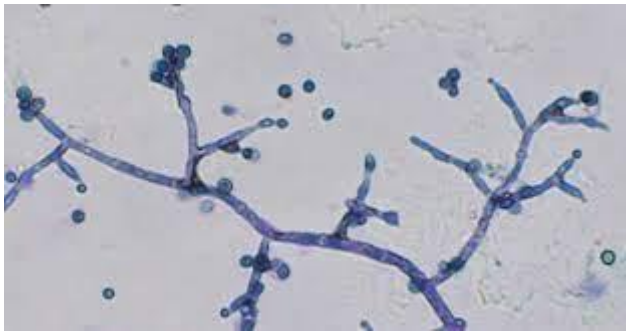
Order 1: *Moniliales*

Family: *Moniliaceae*

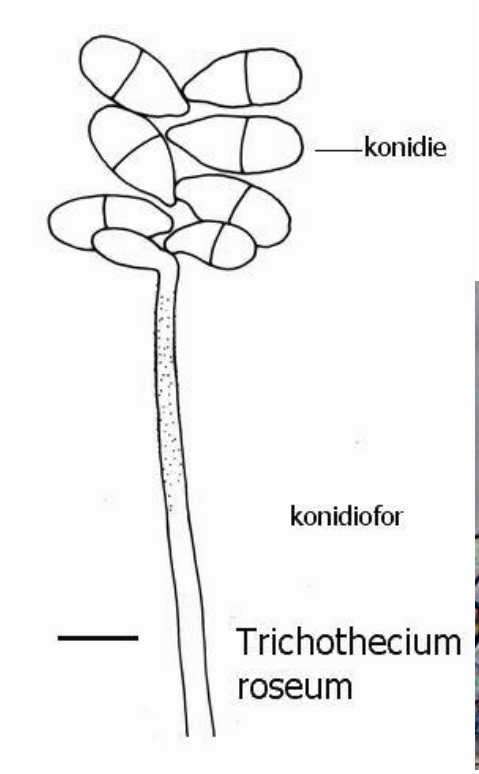
Ex. 1: *Trichoderma viride*

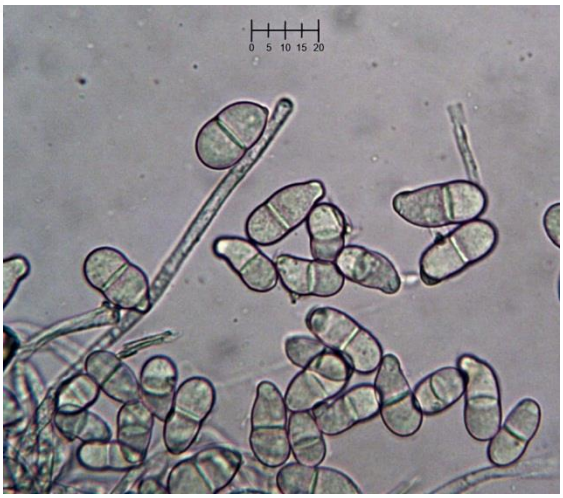
Trichoderma hamatum



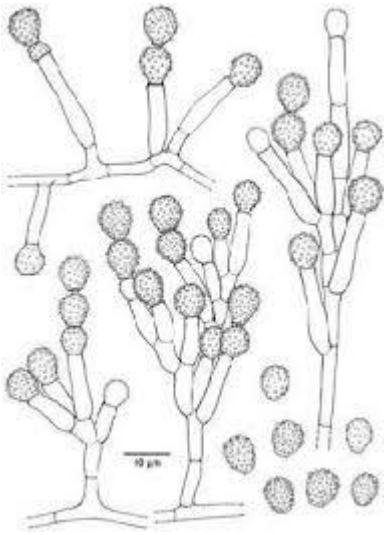


Ex. 2: *Trichothecium roseum*

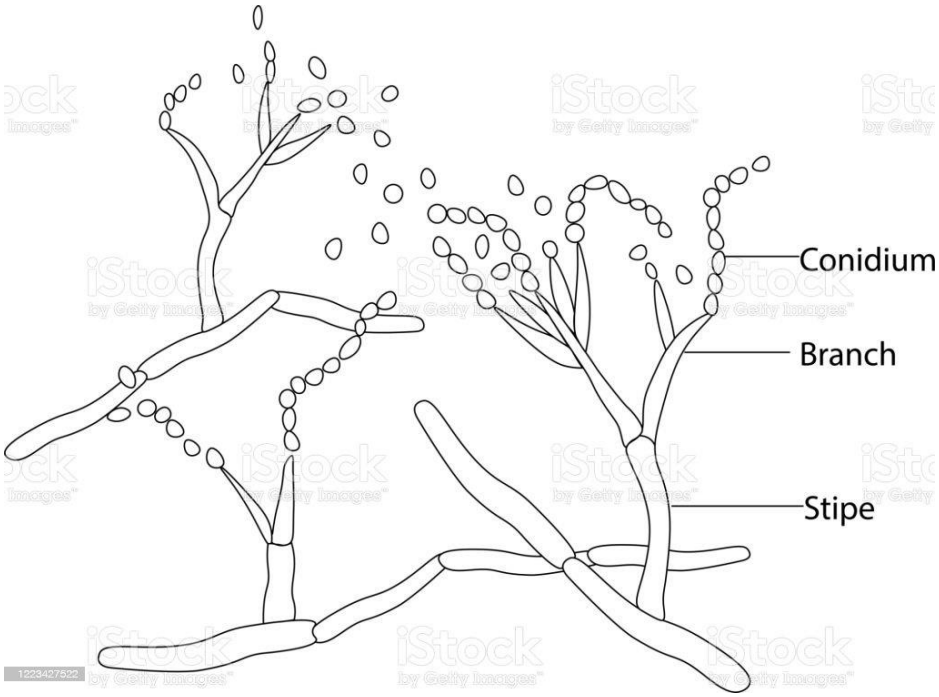




Ex. 3: *Scopulariopsis brevicaulis*

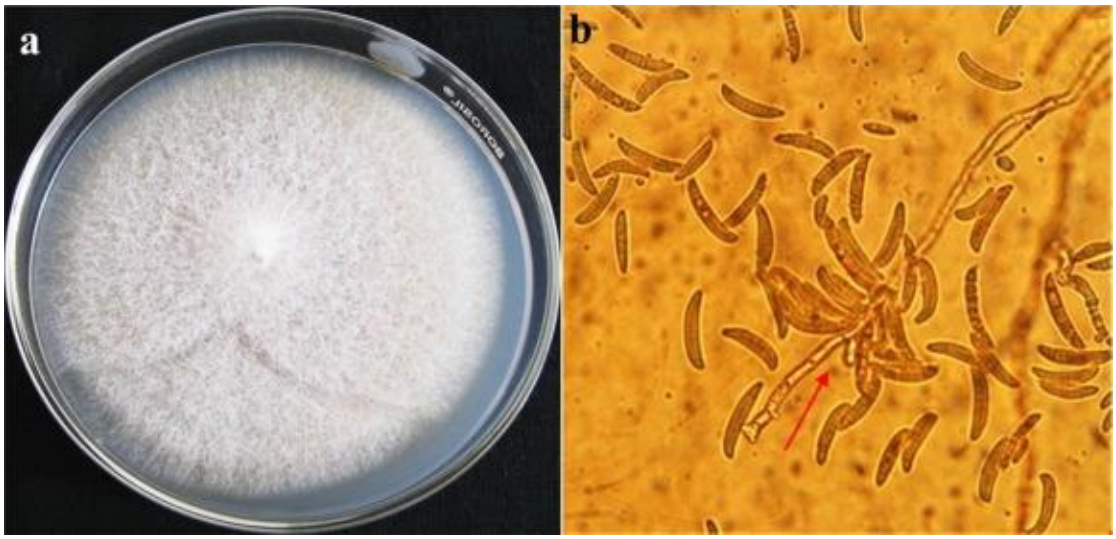


Ex. 4: *Pacielomyces variotii*

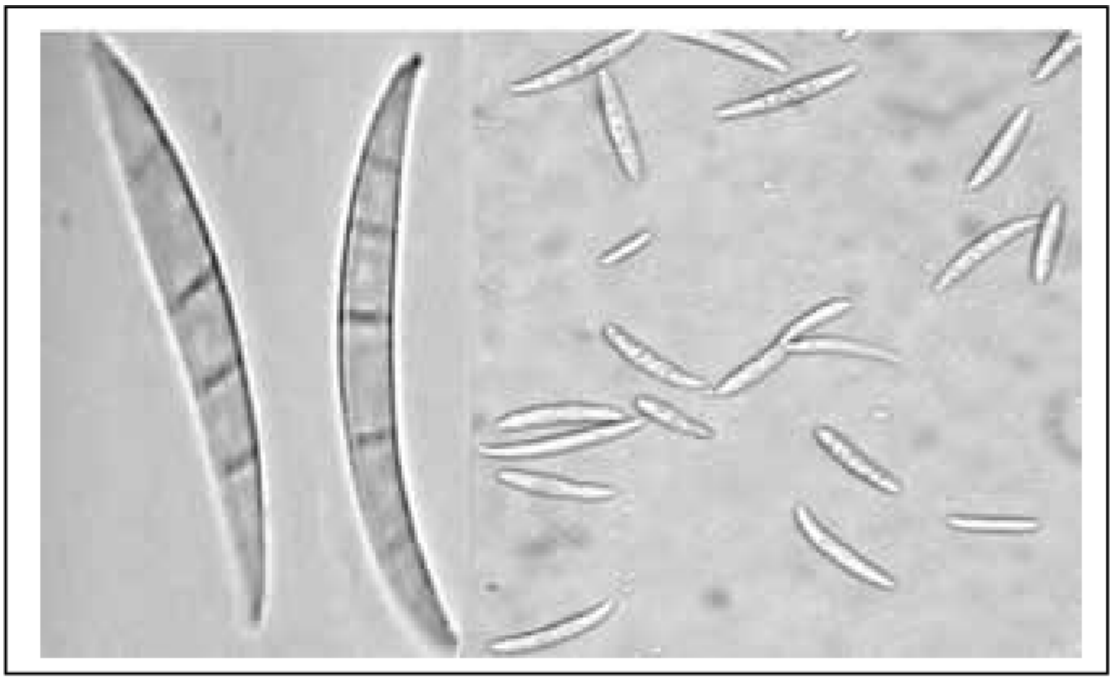


Family 2: *Tuberculariaceae*

Ex. 1: *Fusarium oxysporum*



F. solani

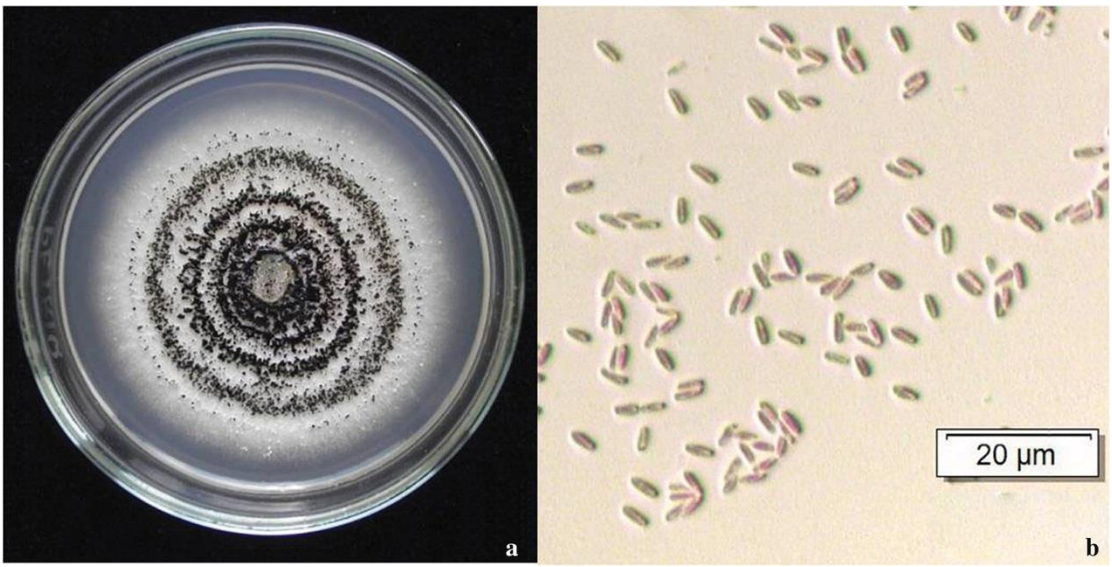


F. moniliform

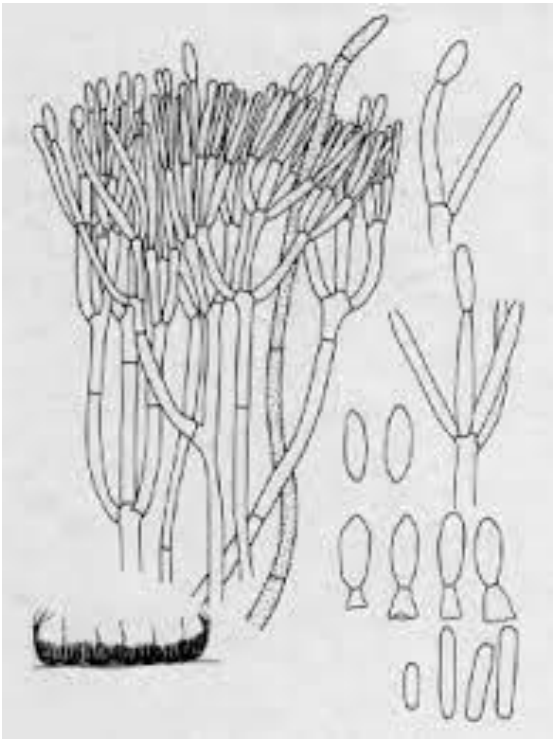
F. merismioides

F. tricinctum

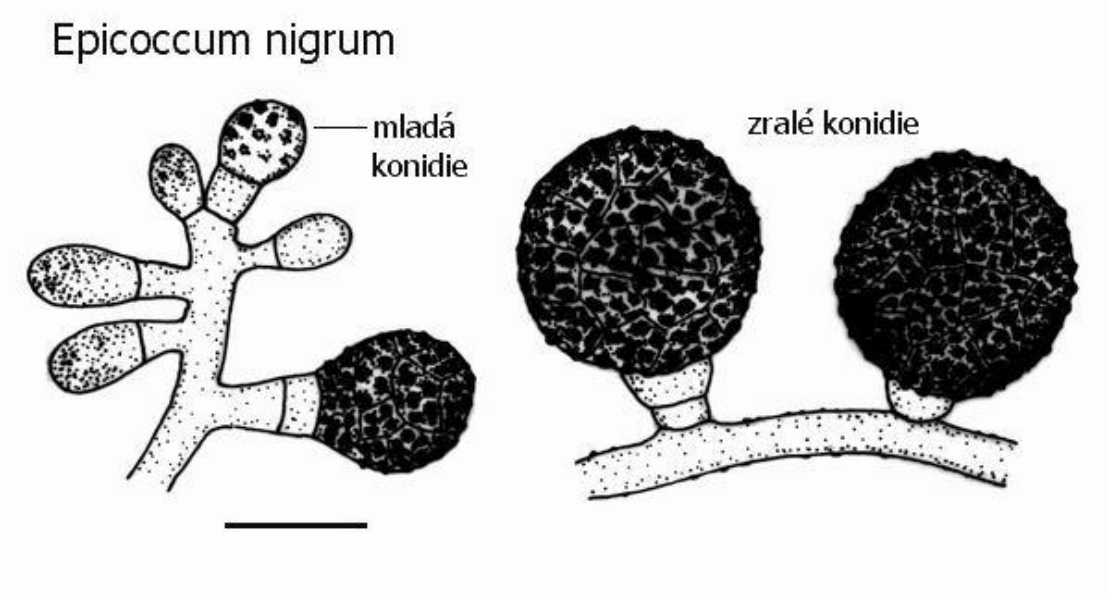
Ex. 2: *Myrothecium roridum*



M. verrucaria

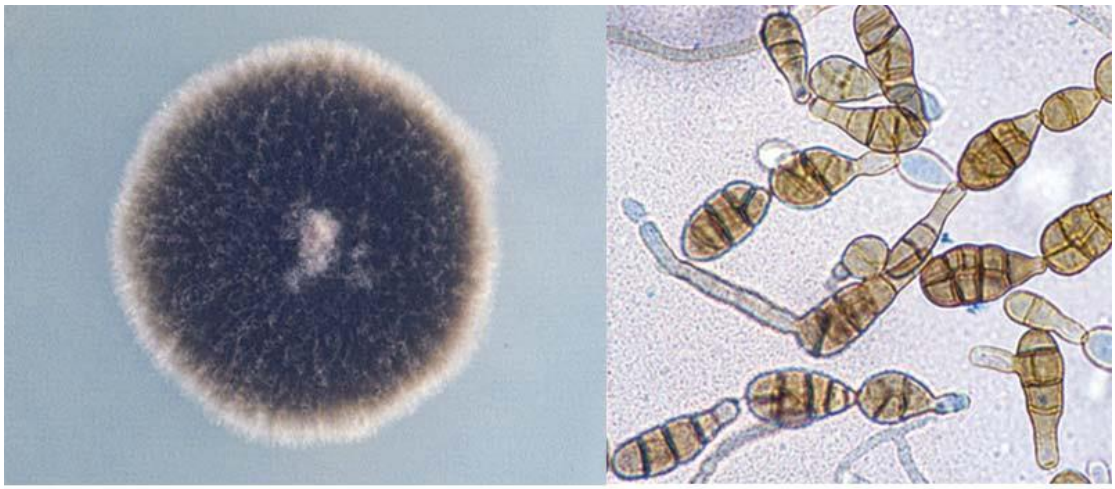


Ex. 3: *Epicoccum nigrum*



Family 3: *Dematiaceae*

Ex. 1: *Alternaria alternate*



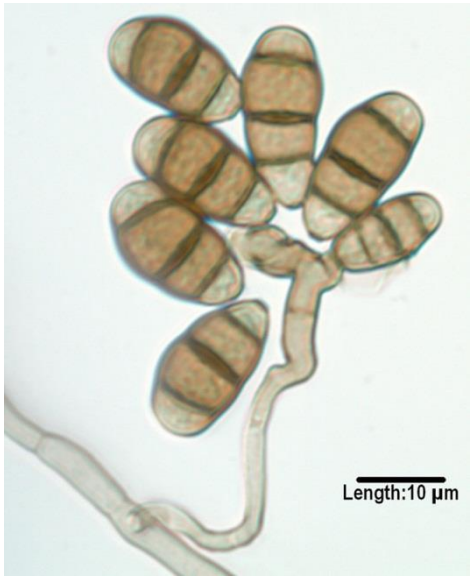
Alternaria alternata **colonies are black to olivaceous-black or greyish, and are suede-like to floccose.**

Alternaria alternata **showing branched acropetal chains and multicelled, obclavate to obpyriform conidia with short conical beaks.**

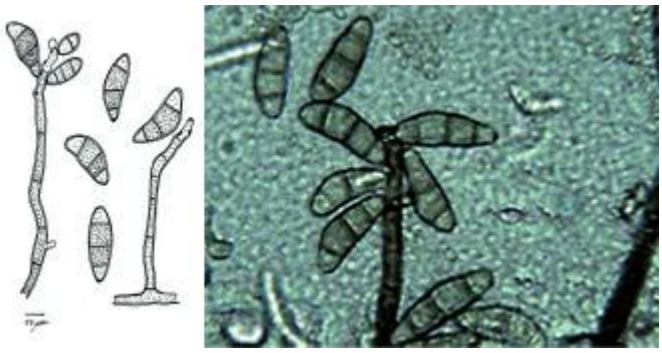
- *A. chlamydospora*
- *A. citri*

Ex. 2: *Ulocladium chartarum*

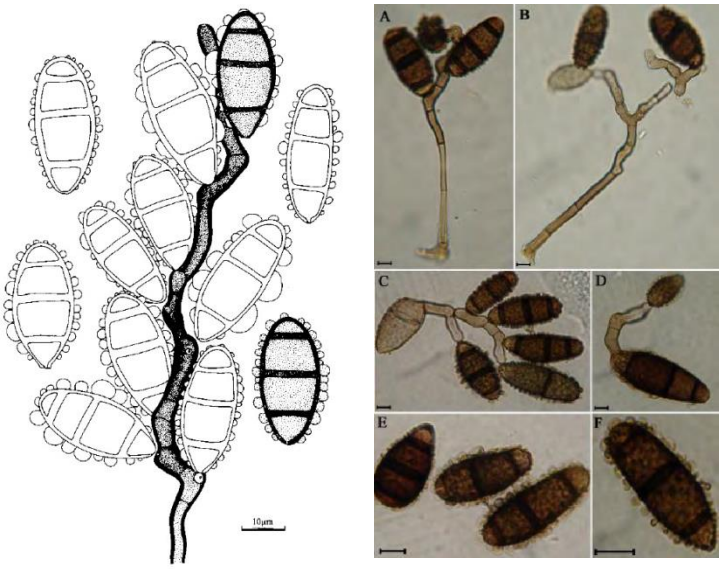
Ex. 3: *Curvularia lunata*



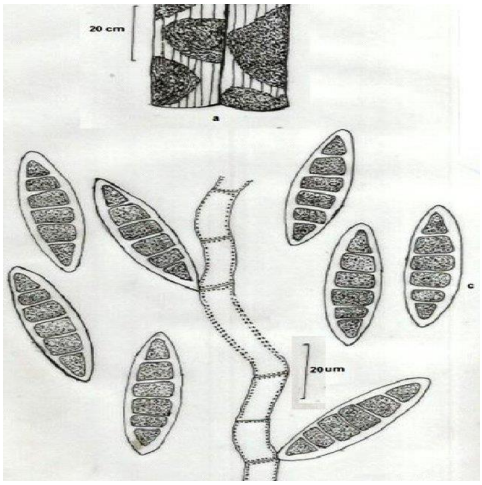
C. pallescens



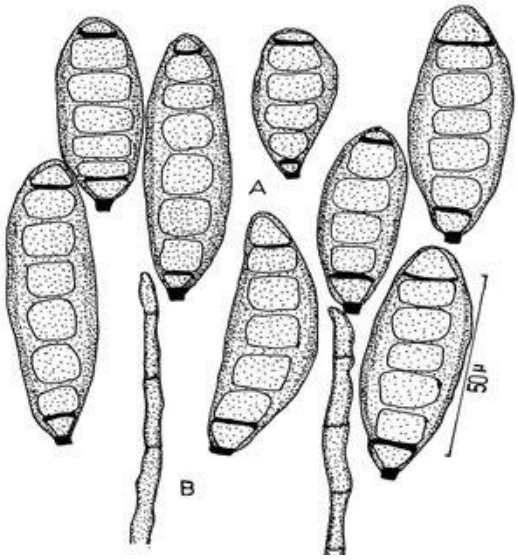
C. tuberculata



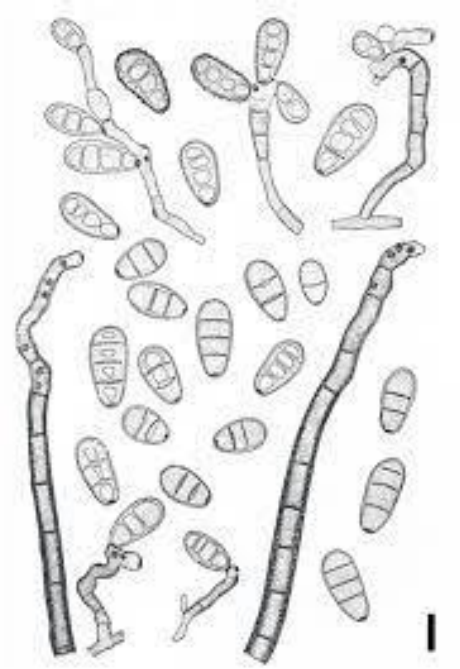
Ex. 4: *Drechslera indica*



D. rostrata



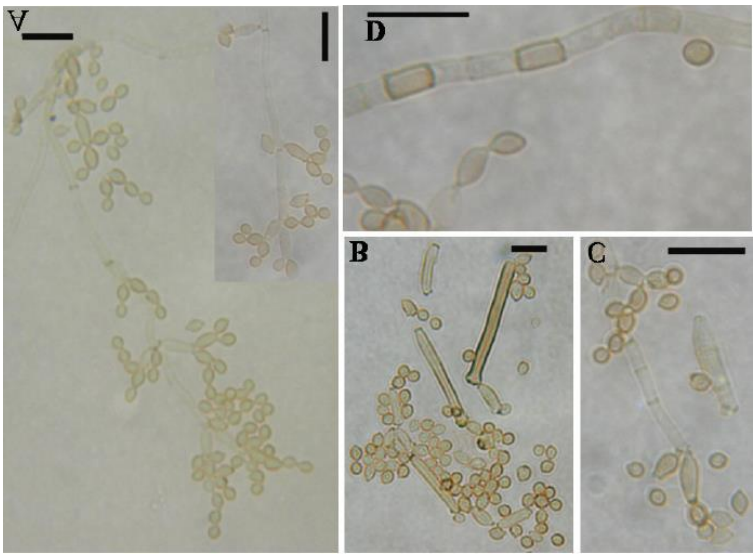
D. biseptata



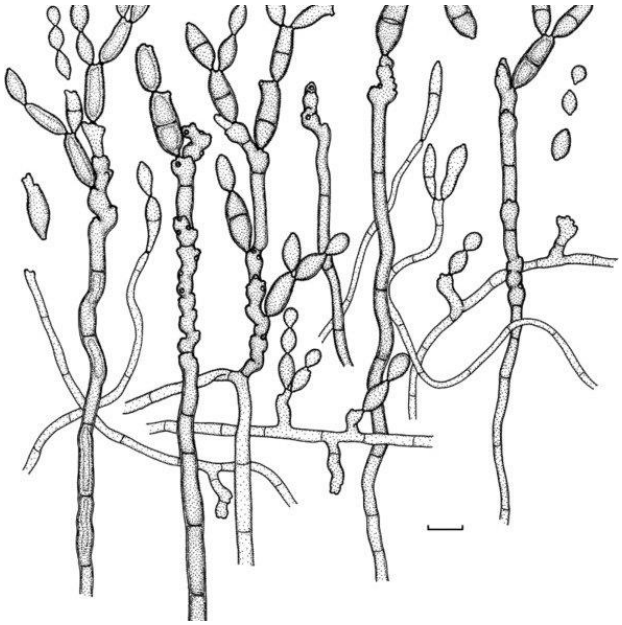
Ex. 5: *Stachybotrys chartarum*

Ex. 6: *Cladosporium cladosporioides*

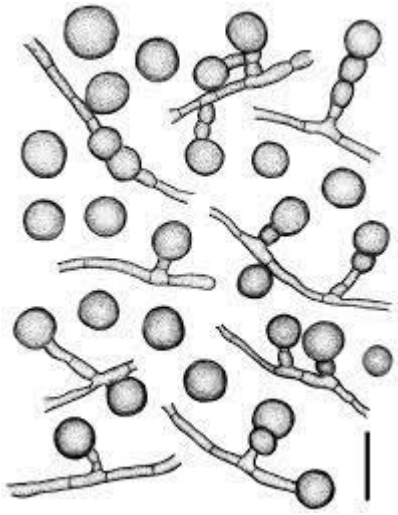
C.sphaerospermum



C.herbarum



Ex. 7*: Humicola grisea*



H. stellate الكونيده نجمية الشكل (

Family 3: *Stilbaceae*

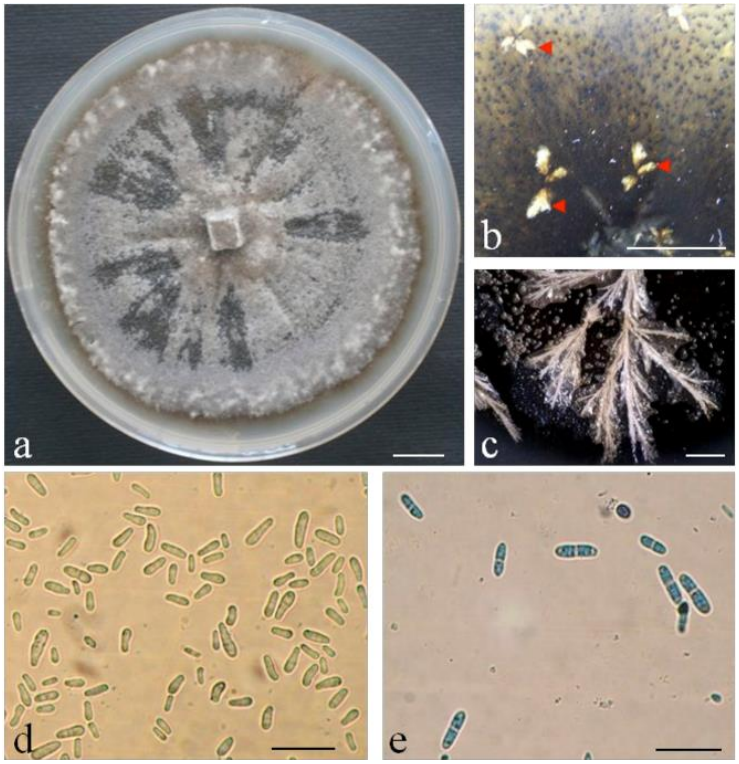
Ex. *Trichorus spirales*

Order 2: Sphaeropsidales

Family: Sohaeropsidaceae

Ex. : *Phoma eupyrena*

P. medicaginis



P. exigua

P. cylindrospora