CULTURE TECHNIQUES
STERILIZATION

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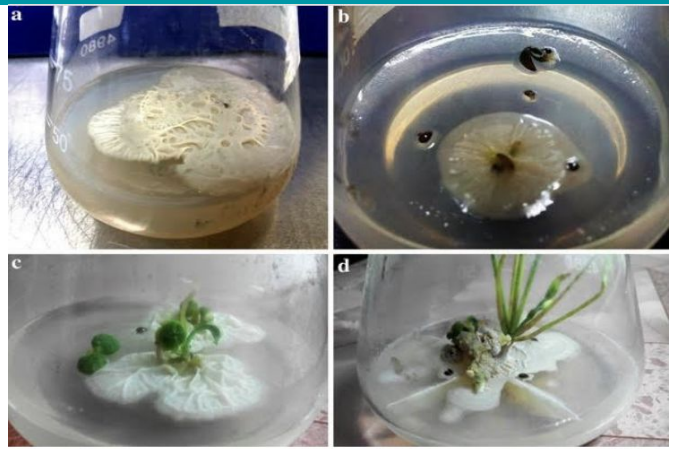
Microbes/ fungi/ Plant tissues can be cultured on a nutrient culture medium under sterile (aseptic) and controlled environmental conditions.

Aseptic technique- Technique by which occurrence of infection into an uninfected tissue is prevented.

Contamination in Tissue Culture

Contamination in tissue culture can originate from two sources-

- through carry over of microorganisms on the surface or in the tissues of explants, or
- through faulty procedures in the laboratory.



https://link.springer.com/article/10.1007/s11738-018-2727-3

https://www.pinterest.com/pin/

Biological Contaminants

Microorganisms are responsible for contamination/ infection.

Different biological contaminants plant tissue culture processes are:

- Bacteria
- Fungi
- Viruses
- Mycoplasmas
- Cross-contamination from other cell lines

Sources of Infection in Cultures

- Glassware and Plasticware
- Culture medium
- Plant material
- Environment in the laboratory
- Instruments used for culture
- Person working in the lab

Non Sterile instruments, supplies, media, or solutions during routine cell culture procedures and non sterile working area are the major source of contamination/ infection.

Sterilization

Sterilization is a process that eliminates (removes) or kills (deactivates) all forms of life and other biological agents with the use of either physical or chemical agents.

Sterilization is a process by which an article, surface or medium is made free of all microorganisms either in the vegetative or spore form.

Disinfection is a process in which there is destruction of all pathogenic organisms capable of producing infection but not necessarily spore forms.

Sterilization

Sterilization is distinct from disinfection, sanitization, and pasteurization, in that those methods reduce rather than eliminate all forms of life and biological agents present.

Pasteurization is the partial sterilization of a product, such as milk or wine, to make it safe for consumption and improve its shelf life.

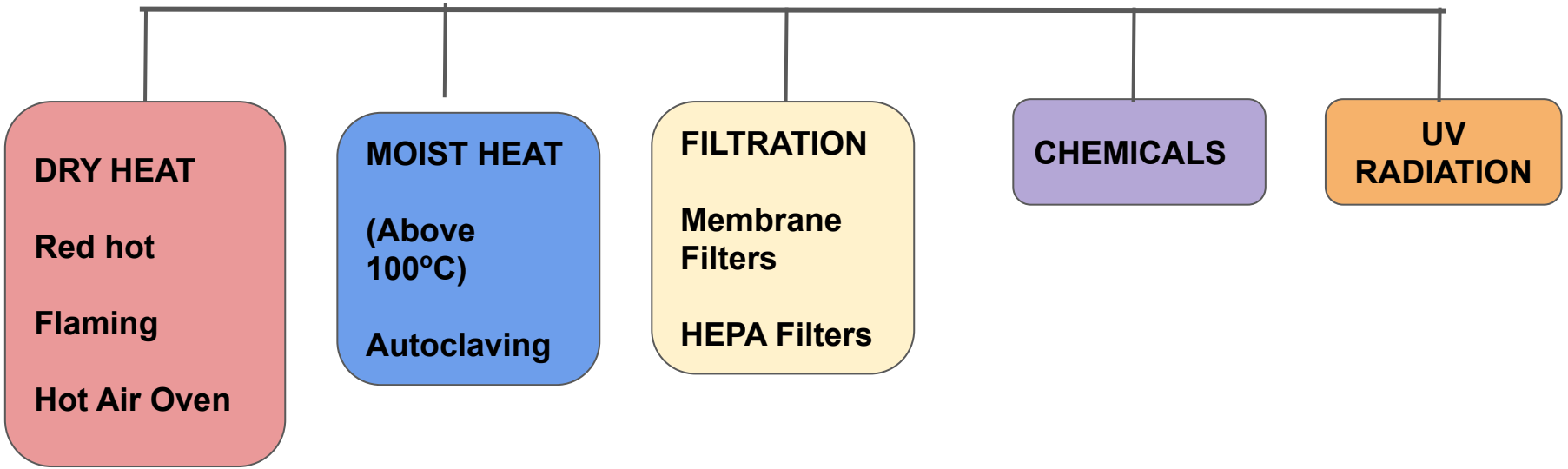
Need for sterilization

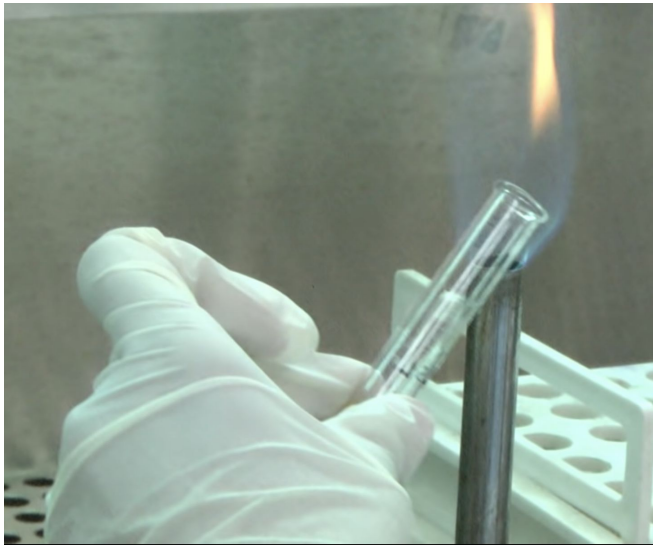
The tissue culture medium is

- rich in sugar, organic and inorganic nutrients.
- supports good growth of microorganisms.
- microorganisms may grow faster than the plant tissues, finally killing them.
- The microbes may also secrete toxic wastes inhibiting growth of cultured tissues.

It is essential to maintain a completely **aseptic environment** inside culture vessels.

Methods of sterilization used in Tissue Culture Labs





Red heat- wire loops, needles, tips of forceps are held in the flame of Bunsen Burner till they become red hot

Flaming- Mouths of culture tubes and flasks are flamed without allowing them to become red hot.

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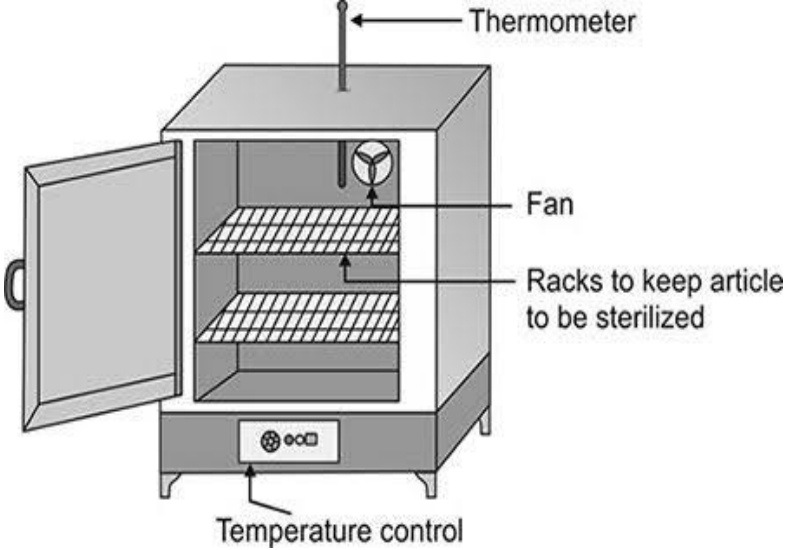


https://www.jaypeedigital.com/eReader/chapter/9789385891540/ch9

Hot air Oven is electrically heated and fitted with a fan for even distribution of hot air in the chamber.

Fitted with a thermostat that maintains the chamber air at a chosen temperature.

It is the most widely used method for sterilization by dry heat.

Used for sterilization of glassware, instruments

Dry Heat Kills the microorganisms by-

- Denaturation of bacterial proteins
- Oxidative damage
- Toxic effect of elevated levels of electrolytes
- Damage to microbial DNA

Sterilization Conditions for HOT Air Oven

Temperature	Hold Time
160°C	120 min
170°C	60 min
180°C	30 min

Time required for sterilization is inversely proportional to temperature to which organisms are exposed.

Precautions while using Hot Air Oven

- Should not be overloaded.
- Glasswares should be arranged in such a manner to allow free circulation of air.
- Materials to be sterilized should be completely dry.
- Test tubes, flaske to be sterilized should be fitted with a cotton plug.
- Petri dishes, pipettes, instruments should be wrapped in a paper.
- Rubber material (except silicone rubber) and inflammable material should not be kept in an oven.
- Oven must be allowed to cool before opening since glassware may crack by sudden cooling.

Moist Heat Sterilization

Pasteurization of milk (Below 100^{o}C)

Intermittent Sterilization/ Tyndallization

Steam at 100^{o}C for 20 minutes for 3 successive days.

Principle- Ist exposure kills all vegetative forms, remaining spores germinate into vegetative forms in the intervals between the heating and are killed by subsequent heating.

It is used for sterilization of media that are destroyed by higher temperature.

Moist Heat Sterilization (above 100ºC)- Autoclaving

At normal atmospheric pressure water boils at 100ºC (When its vapour pressure becomes equal to atm pressure).

When pressure inside the closed vessel increases , temperature at which the water boils also increases.

In autoclave saturated steam at high temperature is used for sterilization.

Saturated steam above 100ºC can penetrate easily and has better killing power as bacterial proteins coagulate rapidly.

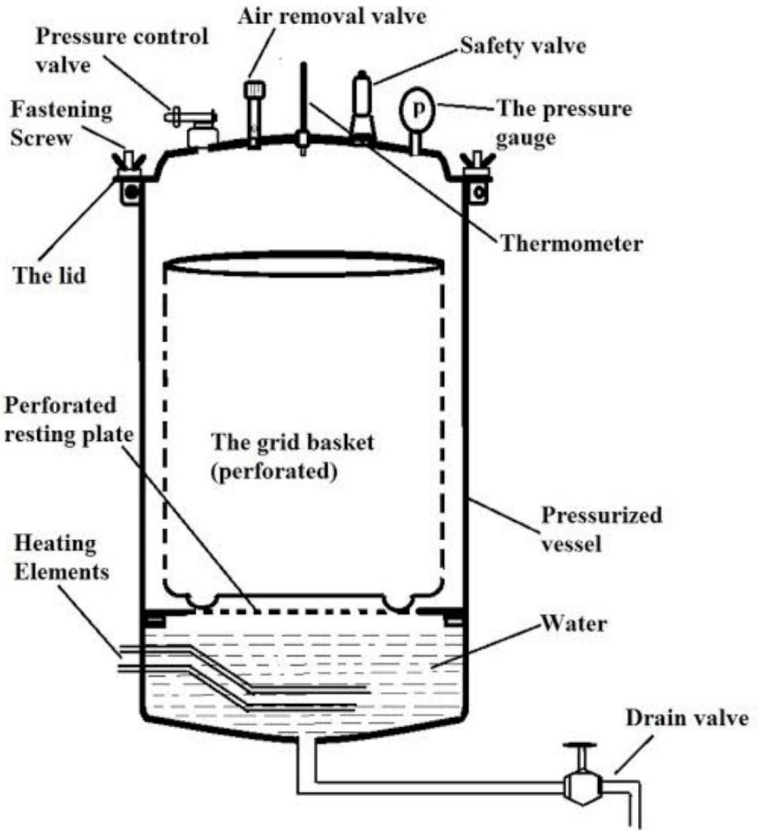
Autoclave

Autoclave is a modified pressure cooker or a boiler that kills microorganisms including heat-resistant endospores using saturated steam under pressure.

https://m.indiamart.com/proddetail/horizontal-autoclave-machine-17505072548.html

https://m.indiamart.com/proddetail/stainless-steel-vertical-autoclave-14235723888.html

At 15 pounds/square inch (lb/in2)] steam pressure and temperature of 121°C., the time of autoclaving to achieve sterilization is generally considered to be 15-20 min.



https://www.microscopemaster.com/autoclave.html

Autoclave comprises three parts: a pressure chamber, a lid, and an electrical heater heats the water to produce steam.

Pressure chamber consists of –
Large cylinder (vertical or horizontal) in which the materials to be sterilized are placed. It is made up of gunmetal or stainless steel and placed in a supporting iron case through a steam jacket (water compartment).

The **lid** is fastened by screw clamps and rendered airtight by an asbestos washer. Lid bears-
A discharge tap for air and steam discharge
A pressure gauge (sets the pressure at a particular level)
A safety valve (to remove the excess steam)

PROCEDURE

1. Place the material to be sterilized inside the pressure chamber and fill the cylinder with sufficient water.
2. Close the lid and put on the electrical heater.
3. Adjust the safety valve to the required pressure.
4. After the water boils, allow the steam and air mixture to escape through the discharge tap till all the air has been displaced
5. This can be tested by passing the steam-air mixture liberated from the discharge tap into a pail of water through a connecting rubber tube. When the air bubbles stop coming in the pail, it indicates that all the air has been displaced by steam.
6. Close the discharge tap.
7. The steam pressure rises inside and when it reaches 15 pounds (lbs) per square inch , the safety valve opens and excess steam escapes out.
8. Holding period from this point of time is about 15-20 minutes .
9. After the holding period, stop the electrical heater and allow the autoclave to cool until the pressure gauge indicates that the pressure inside is equal to the atmospheric pressure.
10. Open the discharge tap.
11. Open the lid of the autoclave and remove the sterilized materials.

Autoclave - Uses

To sterilize culture media, distilled water, gloves, gowns, rubber materials.

Precautions-

Air must be allowed to escape the chamber as temperature of air-steam mixture is less than the pure steam.

Materials should be arranged in such a manner as to ensure free circulation of steam inside the chamber.

Membrane Sterilization

Filtration is the sterilization method that eliminates bacteria by separating the microorganisms from the sterilized medium.

It is an effective method of sterilization for heat sensitive liquids.

Filtration uses membrane filter of pore size 0.22-0.45 micro m, that prevent bacteria to pass through the filter.

Membrane filters are made of cellulose acetate, cellulose nitrate,Polyethersulfone etc.

https://www.fishersci.ca/shop/products/non-sterile-millex-syringe-filter-8/p-7139519

Syringe Filter Unit

with a 0.22 µm pore size hydrophilic Polyethersulfone (PES) membrane.

https://www.cheimika.it/en-US/articles/Vacuum-filter-system-HC-Series-7403.aspx

Vacuum filtration Unit

HEPA Filters & UV Light

HEPA filter "high efficiency particulate air [filter]" can remove at least 99.97% of dust, pollen, mold, bacteria, and any airborne particles with a size of 0.3 microns (μm).

A HEPA filter is a major part of a laminar flow cabinet.

Laminar flow cabinets may have a UV germicidal lamp to sterilize the interior before usage to prevent contamination of the experiment.

https://m.indiamart.com/proddetail/horizontal-laminar-air-flow-chamber-1236214412.html

Laminar Air Flow Cabinet/ Hood

Laminar Airflow Cabinet

Laminar airflow cabinets are used to carry out aseptic manipulations.

A laminar airflow cabinet has a fan.

The dust-free air, which is under pressure, gets pushed through a fine filter, known as the ‘‘High Efficiency Particulate Air (HEPA)’’ filter.

The HEPA filter prevents the entry of particles larger than 0.3 micro m.

The ultra clean air, free of fungal and bacterial contaminants, flows through the working area

Germicidal UV lamps are usually kept on for fifteen minutes to sterilize the interior before the cabinet is used. UV lamps are switched off when the cabinet is being used.

Glassware and Plasticware

- Soaked overnight in a laboratory detergent and scrubbed with a bottle brush.
- Rinsed under tap water followed by distilled water.
- Dried agar - removed by heating.

The contaminated glass and plastic culture vials should be autoclaved before washing or discarding to minimize the spread of bacterial and fungal contaminants in the laboratory.

Culture vials may be sterilized by autoclaving or dry heating in an oven at 160–180 °C for 3 h.

Sterilization of Culture Medium

The culture vials containing medium and closed with a suitable bacteria-proof closure are autoclaved at 15 psi and 121 °C for 15–40 min from the time the medium reaches the required temperature and pressure.

Care should be taken to cover cotton plugs with aluminium foil before autoclaving.

For sterilization of small quantities of medium a pressure cooker, which works on the same principle as an autoclave, may be used.

Sterilized culture medium should be incubated at 30–35 °C for 24–48 h before use to ensure that it is free of contaminants.

Thermolabile Compound

Thermolabile compounds are filter-sterilized.

The solution of the thermolabile compound is sterilized by membrane filtration and added to the autoclaved medium cooled to 50–40 °C or to room temperature

For filter-sterilization of a solution, bacteria-proof membranes of pore size 0.22–0.45 micro m are used.

The filter membrane is placed into filter holders of appropriate size and sterilized by autoclaving after wrapping in aluminium foil.

Sterilization of Instruments

Forceps, scalpels, needles, and spatula are sterilized before use by wrapping in aluminium foil and autoclaving.

During aseptic manipulation the instruments are sterilized several times by dipping in 95 % ethanol and flaming and used after cooling.

Glass bead sterilizers (Steripot) are also used for sterilizing instruments.

Embedding the instruments in the heated beads (at around 250 °C) for 5–7 min is adequate to sterilize them.

Infrared sterilizers are also available for sterilizing instruments in the hood.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Sterilization of Plant Tissues

Plant tissues can be surface sterilized using various sterilants.

After the sterilization treatment the plant material is washed 2–3 times in sterilized distilled water in an aseptic area (laminar airflow chamber) to remove any traces of the toxic sterilant.

Sterilization of Plant Tissues

Sterilizing agent	Concentration (%)	Duration (min)	Effectiveness
Calcium hypochlorite	9–10	5–30	Very good
Sodium hypochlorite	2[b]	5–30	Very good
Hydrogen peroxide	10–12	5–15	Good
Bromine water	1–2	2–10	Very good
Silver nitrate	1	5–30	Good
Mercuric chloride	0.1–1	2–10	Satisfactory
Antibiotics	4–50 mg L^{-1}	30–60	Fairly good

Sterilization of Explant

Delicate tissues, such as immature embryos, endosperm, nucellus and shoot tips are sterilized along with the surrounding tissues, and the explant is dissected out under aseptic conditions.

Inoculation of the plant material on the medium is done in the laminar airflow cabinet .

The sterilized plant material or the plant material to be subcultured is placed on a pre-sterilized ceramic tile, steel tray or Petri plate for cutting to proper size before inoculation.

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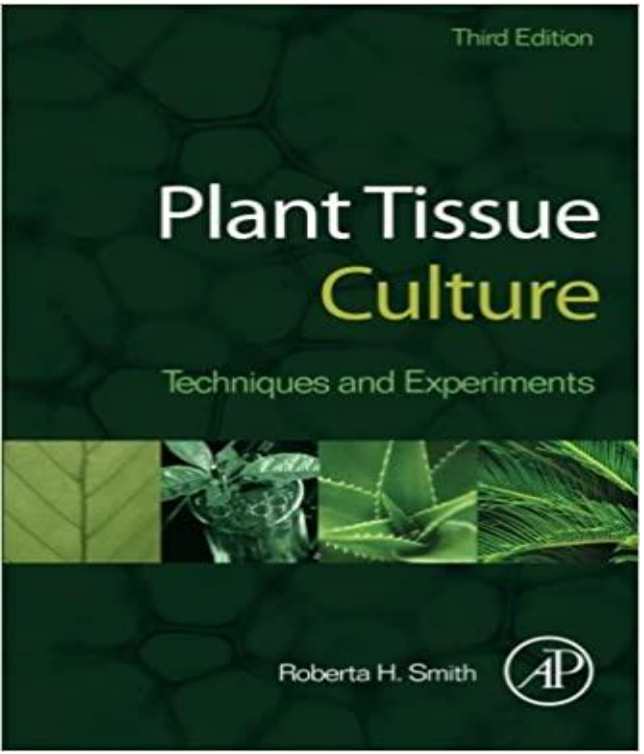
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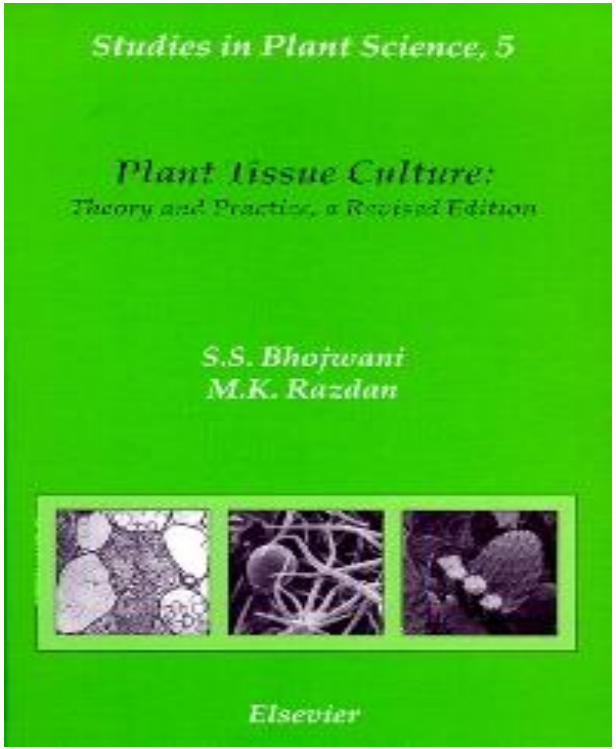
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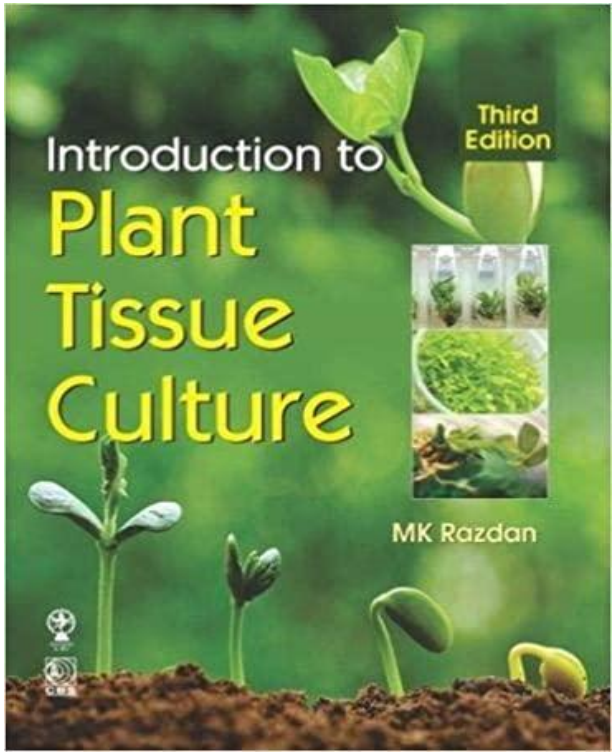
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