DEPARTMENT OF BOTANY
Guru Ghasidas Vishwavidyalaya, Bilaspur
B. Sc. V Semester
LBC – 503 (Plant Tissue Culture)

Section A

Multiple choice questions / Define the following terms **1 × 10 = 10**

1. **(d)** ***Agrobacterium***
2. **(a) Increasing level of cytokinin to a callus induces shoot formation and increasing level of auxin promote root formation**
3. **(d) All of the above**
4. **(d) surface sterilization**
5. **(d) all of above**

1. Primordium

An organ or a part in its most rudimentary form or stage of development or the first recognizable, histologically differentiated stage in the development of an organ.

2. Indirect organogenesis

Explant → Callus → Meristemoid → Primordium

• Dedifferentiation
– Less committed,
– More plastic developmental state

• Induction
– Cells become organogenically competent and fully determined for primordia production

• Differentiation

3. Explants

Plant tissue cultures are initiated from tiny pieces, called **explants**, taken from any part of a plant. The “explant” is removed surgically; its surface is then sterilized and placed on a nutrient medium to initiate the mother culture that is multiplied repeatedly by sub culturing. The following plant parts are extensively used in commercial micropropagation.

4. Protoplast fusion

Protoplast fusion, is a type of genetic modification in plants by which two distinct species of plants are fused together to form a new hybrid plant with the characteristics of both, a somatic hybrid.

5. Somatic embryogenesis

Somatic embryogenesis is a process where a plant or embryo is derived from a single somatic cell or group of somatic cells. Somatic embryos are formed from plant cells that are not

normally involved in the development of embryos, i.e. ordinary plant tissue. No endosperm or seed coat is formed around a somatic embryo.

Section B

Descriptive answer type questions (attempts any four) **4 × 5 = 20**

2. Define cellular totipotency. What are the nutritional requirements for explants to be totipotent and importance of totipotency in tissue culture?

- Totipotency: When plant cells and tissues are cultured in vitro they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type.
- In this way whole plants can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells when given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called 'totipotency'.
- Many somatic plant cells, including some fully differentiated types (e.g. leaf mesophyll), provided they contain intact nuclear, plastid and mitochondrial genomes, have the capacity to regenerate into whole plants. This phenomenon is totipotency.
- It was first demonstrated by Steward and Reinert in the 1950s.
- A differentiated plant cell that is selectively expressing its genetic information can instead initiate expression of the program required for generation of an entire new plant.
- Many plants have been regenerated from single cells, but not all plant cells are totipotent; some are terminally differentiated, often because of partial or complete genome loss.
-

3. Comment on the followings:

(a) Hardening or acclimatization process

- Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus.
- During long term culture, the culture may lose the requirement for auxin and/or cytokinin. This process, known as habituation, is common in callus cultures from some plant species (such as sugar beet).
- Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced.
- Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies.

(b) Shoot tip culture

- ✓ Shoots develop from a small group of cells known as shoot apical meristem.
- ✓ The cells of apical meristem maintains itself and gives rise to new tissues and organs and communicates signals to the rest of the plant.

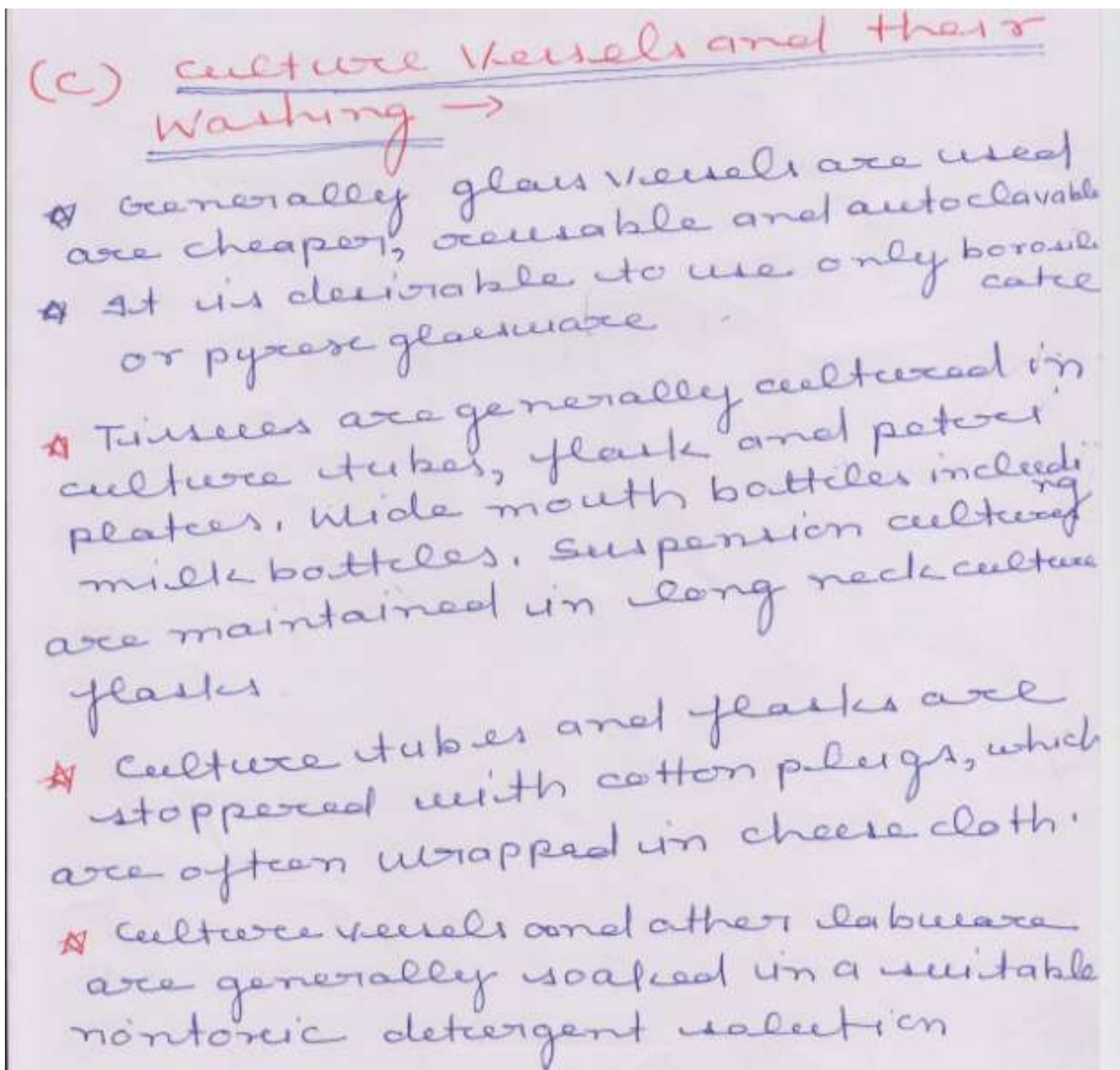
- ✓ Shoot-tips and meristem-tips are perhaps the most popular source of explants to initiate tissue cultures.
- ✓ Production of virus free germplasm
- ✓ Mass production of desirable genotypes
- ✓ Facilitation of exchange between locations (production of clean material)
- ✓ Cryopreservation (cold storage) or *in vitro* conservation of germplasm

4. Describe the plant tissue culture techniques under followings heads

(a) Culture vessels

(b) Sterilization

(c) Culture techniques


(c) Culture vessels and their Washing →

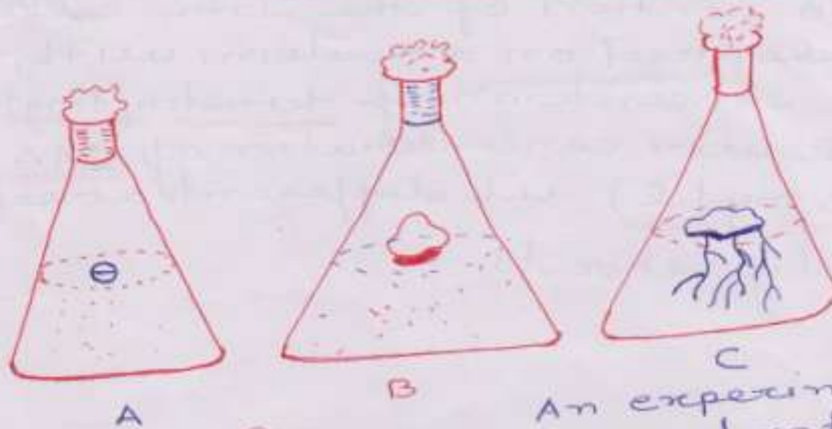
- Generally glass vessels are used are cheaper, reusable and autoclavable
- It is desirable to use only borosilicate or pyrex glassware
- Tissues are generally cultured in culture tubes, flask and petri plates, wide mouth bottles including milk bottles. Suspension cultures are maintained in long neck culture flasks
- Culture tubes and flasks are stoppered with cotton plugs, which are often wrapped in cheese cloth.
- Culture vessels and other labware are generally soaked in a suitable nontoxic detergent solution

D. Sterilization →

All the materials, medium, plant material etc. used in culture work should be freed from microbes -

Sterilization technique	Materials sterilized
① Dry heat	Empty glassware (culture vessels, pipettes etc) and certain plastic ware (Teflon), instruments like scalpels, forceps, needles etc. (glass bead sterilizers used)
② Flame sterilization	Instruments like scalpel, forceps etc. mouths of culture vessels
③ Autoclaving (121°C at 15 p.s.i for (15-40) min)	Media culture vessels (glassware and several plastic ware), other glass and plastic ware, contaminated cultures
④ Filter sterilization (a) Liquid (membrane filter of 0.45 μm of smaller pore size	Heat labile compounds like GA_3, ABA, Zeatin, urea, Enzymes etc.
(b) Air	Air blown through laminar flow cabinets
Wiping with 70% ethanol	Platform of laminar flow cabinets, hands of the operator etc
Surface sterilization	All plant materials to be cultured

Culture Technique



An experiment to demonstrate the effect of growth regulators on growth and differentiation in plant tissue culture

A → On the nutrient medium lacking a growth regulator the tobacco pith tissue showed very poor growth

B → In the presence of a cytokinin a vigorously growing callus was formed

C → When transferred to a medium containing 3 mg/lit of indole acetic (IAA) and 0.2 mg/lit of (kinetin), this

culture technique

The technique of plant tissue culture enables us to study the cells, tissue or organs by isolating from the plant body and growing aseptically, in suitable containers on an artificial nutrient medium, under environmental conditions. Thus (i) nutrient medium (ii) aseptic condition (iii) Aeration of the tissue

Nutrient medium ⇒

Every tissue and organ has its special requirment for optimal growth. However most of media contain inorganic salts of major and minor elements, vitamins and sucrose. A medium with these ingredients will be referred to as basal medium. some time growth regulators such as auxin, gibberellin and cytokinin may also be added to the basal medium. Growth regulators are required for cell divison and organ regeneration from the cultures. The cultures are usually kept in culture room at about 24°C with some illumination. These all constituents are dissolved in distilled water. If necessary the medium is solidified with 0.8% agar.

⑥ The pH of medium is adjusted around 5.8 slightly acidic. Now equal quantities of the medium are dispersed in culture vials which are usually glass tubes or flasks. The culture vials, containing medium are plugged with non-absorbent cotton wrapped in cheese cloth. Such a closure allows the exchange of gases but does not permit the entry of microorganism in cheese cloth.

Aseptic condition ⇒
The sugar content of nutrient media may support a luxuriant growth of many micro-organisms like bacteria and fungi. It is therefore, extremely important to maintain a completely aseptic environment inside the culture vials.

Micro-organisms can contaminate the medium in at least three ways

(a) Microorganisms present in the medium from begining, may be destroyed by sterilizing the properly culture vials. It can be done by maintaing the temperature at 121°C for about 15 minutes

(4)

(b) The microorganism may also be carried along with tissue that is being cultured. To prevent this the plant material from which tissue is to be excised is surface sterilized.

(c) Finally precaution must also be taken to prevent the entry of microorganisms while the plug of a culture vial is removed to transfer the tissue to the nutrient medium. All operations should be done in an aseptic environment.

Aeration ⇒ Proper aeration of the cultured tissue is also an important aspect. If the tissue grown on the surface of semi-medium it acquires enough aeration

5. Comment on the followings

(a) Significance of callus culture

- Callus cultures are extremely important in plant biotechnology.
- Callus cells are not necessarily genetically homogeneous because a callus is often made from structural tissue, not individual cells.
- Plant callus can differentiate into a whole plant, a process called regeneration, through addition of plant hormones in culture medium.
- Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced.
- Callus is of particular use in micropropagation where it can be used to grow genetically identical copies of plants with desirable characteristics.
- Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies.

(b) Factors affecting single cell culture

- Substrate or liquid (cell culture flask or scaffold material)

- chemically modified plastic or coated with ECM proteins
- suspension culture

- Nutrients (culture media)
- Environment (CO_2, temperature 37^oC, humidity)
  - Oxygen tension maintained at atmospheric but can be varied
- Sterility (aseptic technique, antibiotics and antimycotics)
  - Mycoplasma tested
- **Basal Media**
  - Maintain pH and osmolarity (260-320 mOsm/L).
  - Provide nutrients and energy source.
- **Components of Basal Media**
  - Inorganic Salts
  - Maintain osmolarity
  - Regulate membrane potential (Na^+, K^+, Ca^{2+})
  - Ions for cell attachment and enzyme cofactors
- **pH Indicator – Phenol Red**
  - Optimum cell growth approx. pH 7.4
- **Buffers (Bicarbonate and HEPES)**
  - Bicarbonate buffered media requires CO_2 atmosphere
  - HEPES Strong chemical buffer range pH 7.2 – 7.6 (does not require CO_2)
- **Glucose**
  - Energy Source

6. Give a general account of isolation, characterization, molecular basis and application of somaclonal variations.

- Somaclonal variation is the variation seen in plants that have been produced by plant tissue culture.
- Chromosomal rearrangements are an important source of this variation.
- Somaclonal variation is not restricted to, but is particularly common in, plants regenerated from callus. The variations can be genotypic or phenotypic, which in the latter case can be either genetic or epigenetic in origin.
- Typical genetic alterations are: changes in chromosome numbers (polyploidy and aneuploidy), chromosome structure (translocations, deletions, insertions and duplications) and DNA sequence (base mutations).
- Typical epigenetic related events are: gene amplification and gene methylation.
- If no visual, morphogenic changes are apparent, other plant screening procedures must be applied.
- There are both benefits and disadvantages to somaclonal variation.
- The phenomenon of high variability in individuals from plant cell cultures or adventitious shoots has been named somaclonal variation.

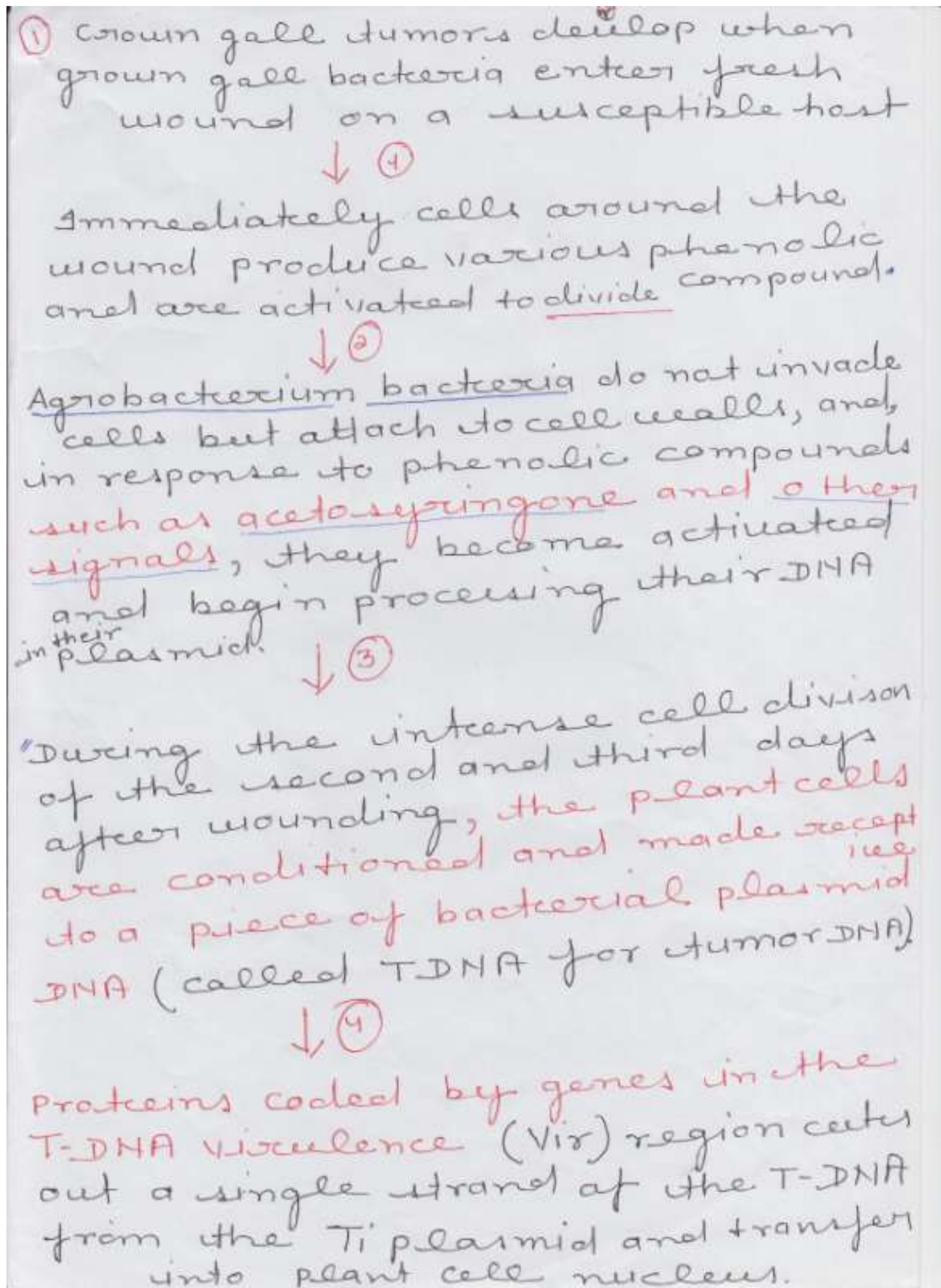
Applications

- The major likely benefit of somaclonal variation in plant is improvement.
- Somaclonal variation leads to the creation of additional genetic variability.
- Characteristics for which somaclonal mutants can be enriched during *in vitro* culture includes resistance to disease pathotoxins, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites.

- ➢ Micropropagation can be carried out throughout the year independent of the seasons and plants.

7. Comment on the followings:

(a) Methods of transformation



① Crown gall tumors develop when crown gall bacteria enter fresh wound on a susceptible host

↓ ①

Immediately cells around the wound produce various phenolic compound. and are activated to divide

↓ ②

Agrobacterium bacteria do not invade cells but attach to cell walls, and in response to phenolic compounds such as acetosyringone and other signals, they become activated and begin processing their DNA in their plasmid.

↓ ③

"During the intense cell division of the second and third days after wounding, the plant cells are conditioned and made receptive to a piece of bacterial plasmid DNA (called T-DNA for tumor DNA)

↓ ④

Proteins coded by genes in the T-DNA virulence (Vir) region cuts out a single strand of the T-DNA from the Ti plasmid and transfer into plant cell nucleus.

Dr. Meenakshi Singh

In crown gall, a disease caused by the bacterium A. tumefaciens starting on more than a hundred plant species, galls or tumors develop on the roots, stems, leaves, ears, tassels and petioles of host plants.

↓ (5)

T-DNA become integrated into nuclear DNA and some of its genes are expressed and lead to synthesis of auxins and cytokinins which transform normal plants cells into tumor cells.

↓ (6)

Tumor cells grow and divide independently of the bacteria, and their organization, rate of growth and rate of division can no longer controlled by the host

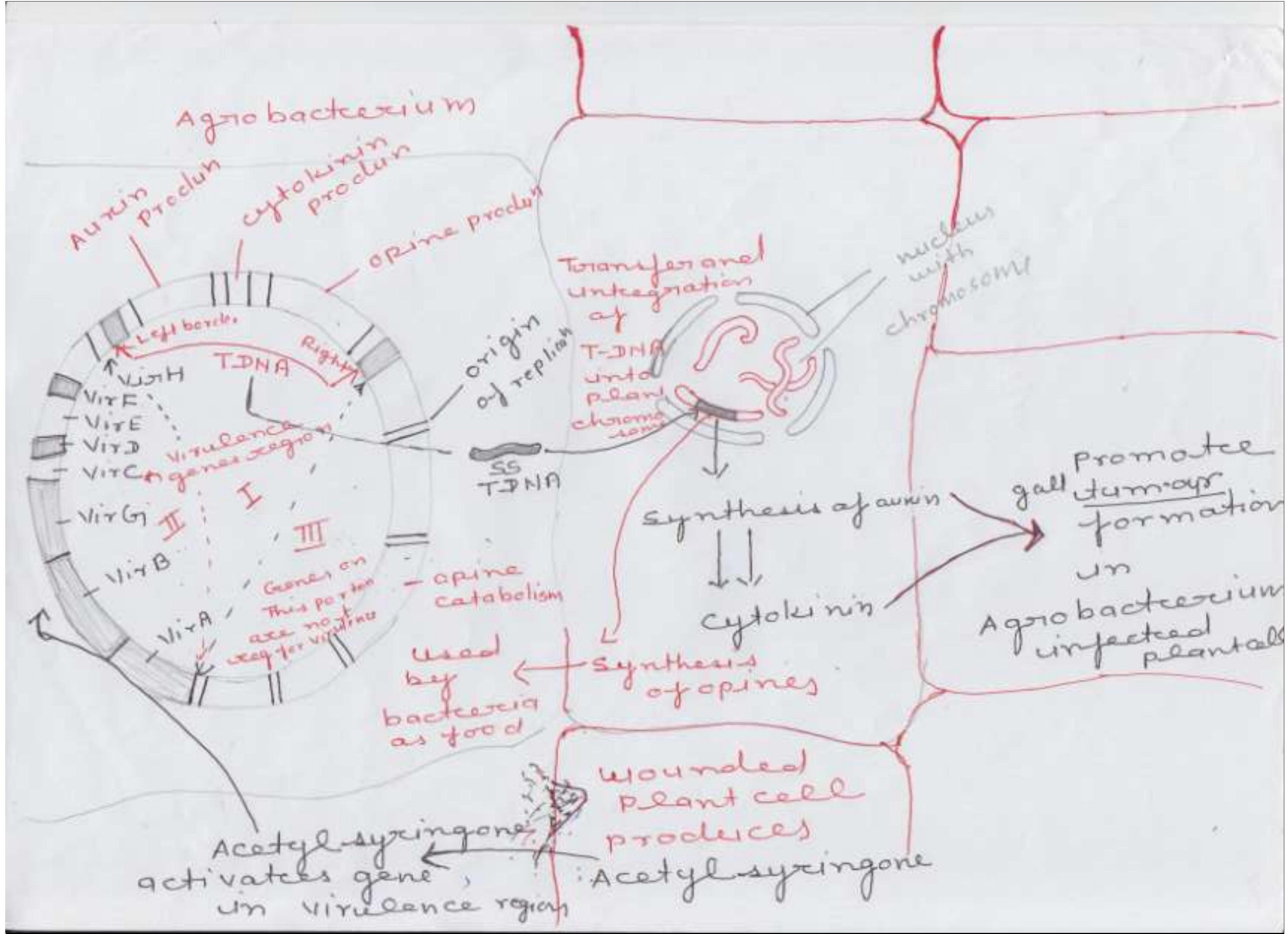
The integrated T-DNA also contain genes that code for substances known as opines, which can used only by the intercellularly growing gall bacteria as a source of food

and development of normal anatomical features in the plantlets raised in vitro. As a result, such plantlets show improved survival on transfer ex vitro.

→ Coculture with Micro-organism

Inoculation of plantlets with a suitable bacterial culture, e.g. Pseudomonas species is called bacterization.

Bacterization with Pseudomonas af potato plantlets significantly increased their root number, root dry weight, stem length, leaf area. In addition bacterized plantlets showed a higher total plant lignin content and improved stomatal function that increased their tolerance to dehydrating conditions.



Agrobacterium
Auxin produn
cytokinin produn
opine produn
Left border
Right border
TDNA
VirH
VirF
VirE
VirD
VirC
VirG
VirB
VirA
Virulence genes region
I
II
III
Genes on this portion are not reqd for virulence
opine catabolism
origin of replication
SS TDNA
Transfer and integration of T-DNA into plant chromosome
nucleus with chromosome
Synthesis of auxin
cytokinin
Synthesis of opines
used by bacteria as food
Promote gall tumour formation in Agrobacterium infected plant cells
wounded plant cell produces
Acetyl-syringone
Acetyl-syringone activates gene in virulence region

(b) Applications of transgenic plants

(i) Improved Nutritional Quality (ii) Insect Resistance
(iii) Disease Resistance (iv) Herbicide Resistance
(v) Salt Tolerance (vi) Biopharmaceuticals

8. Comment on the followings:

(a) Habituation in callus culture

- ✓ During callus formation, there is some degree of dedifferentiation (i.e. the changes that occur during development and specialization are, to some extent, reversed), both in morphology (a callus is usually composed of unspecialized parenchyma cells) and metabolism.
- ✓ This has important consequences for the culture of callus tissue, as the metabolic profile will probably not match that of the donor plant.
- ✓ Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus.
- ✓ During long term culture, the culture may lose the requirement for auxin and/or cytokinin.
- ✓ This process, known as **habituation**, is common in callus cultures from some plant species (such as sugar beet).
- ✓ Callus cultures are extremely important in plant biotechnology.
- ✓ Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced.

(b) Isolation techniques of single cell

A) From Plant Organ:

- ➢ The most material for the isolation of single cell aseptically on a nutrient medium under controlled conditions.

Method of Single Cell Isolation:

i) Mechanical Method

- ➢ Mechanical isolation involves tearing or chopping surface sterilized explant to expose the cells followed by scrapping of the cells with a fine scalpel to liberate the single cells hoping that it remained undamaged.
- ➢ The mechanical isolation of free parenchymatous cells can also be achieved on a large scale.

i) Enzyme Method

- ➢ Isolation of single cells by the enzymatic method has been found convenient, as it is possible to obtain high yields from preparation of spongy parenchyma with minimum damage or injury to the cells.
- ➢ Applying enzymatic method to cereals (*Hordeum vulgare, Zea mays*) has proven rather difficult since the mesophyll cells of these plants are apparently elongated with number of interlocking constrictions, thereby preventing their isolation.

B) From Cultured Tissue:

- ➢ The most widely applied approach is to obtain a single cell system from cultured tissue. Freshly cut piece from surface sterilized plant organs are simply placed on a nutrient medium consisting of a suitable proportion of auxins and cytokinins to initiate cultures.

- Explant on such a medium exhibit callusing at the cut ends, which gradually extends to the entire surface of the tissue.
- The callus is separated from an explant and transferred to a fresh medium of the same composition to enable it to built up a mass tissue.

② Culture of Single Cells

- Single cells can be cultured using the following techniques -
  (i) Filter paper raft - nurse technique
  (ii) Microchamber technique
  (iii) Microdrop method
  (iv) Bergman's plating technique
  (v) Thin layer liquid medium
- Isolated single cell fail to divide in normal tissue culture media. Either a nurse tissue or a conditioned medium is used for their culture medium.

A conditioned medium - is a medium in which plant cells have been grown at a high cell density for about 24-48 hrs after which the cells had been filtered out.

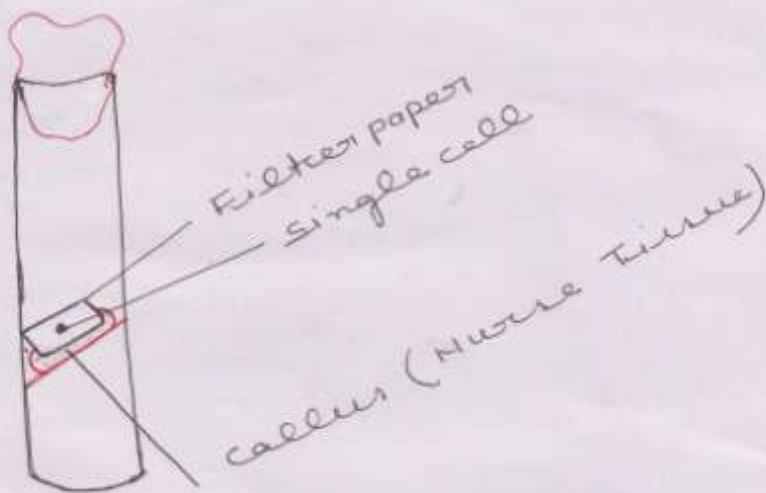
- The conditioned medium is enriched by the various molecules secreted by the cultured plant cells, which sustain proliferation and development of the cells cultured in such a medium at a rather low density.

  proli - rapid increase in the number of cells

- Plant cells in culture release protein and carbohydrates, some of which function as signalling molecules for (while) some others can sustain for embryogenesis and somatic embryo development.

⇒ Filter Paper Raft - Nurse Tissue Technique

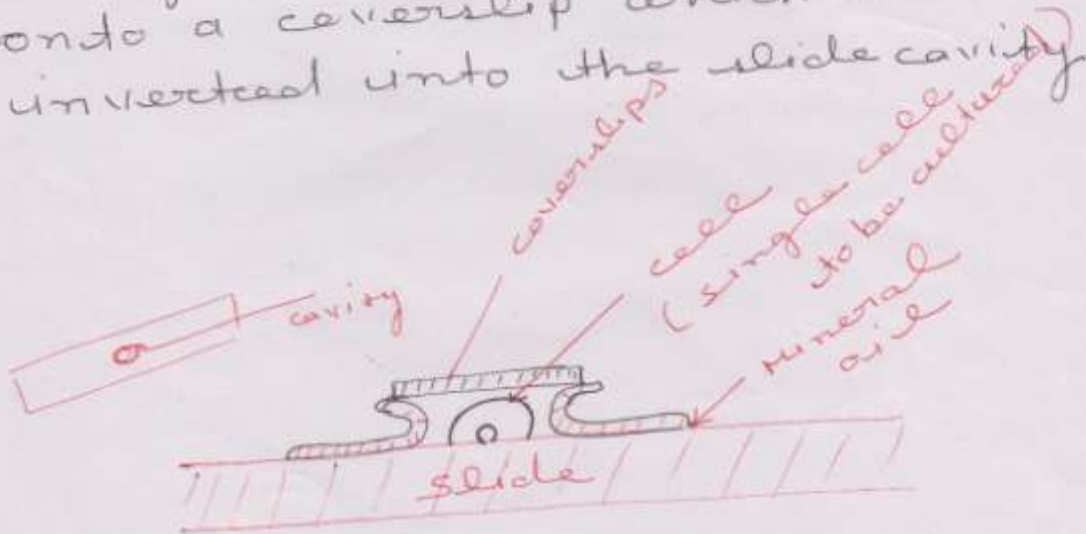
(3)



Single cells are placed on small pieces (8×8 mm) of filter paper (sterilized) which themselves are placed on top of established callus cultures. This allows the filter paper to be wetted by the exudates from callus tissue. The single cells placed on filter papers derive their nutrition from the callus exudates diffusing through the filters. The cells divide and form macroscopic colonies on the filters; the colonies are then isolated and cultured.

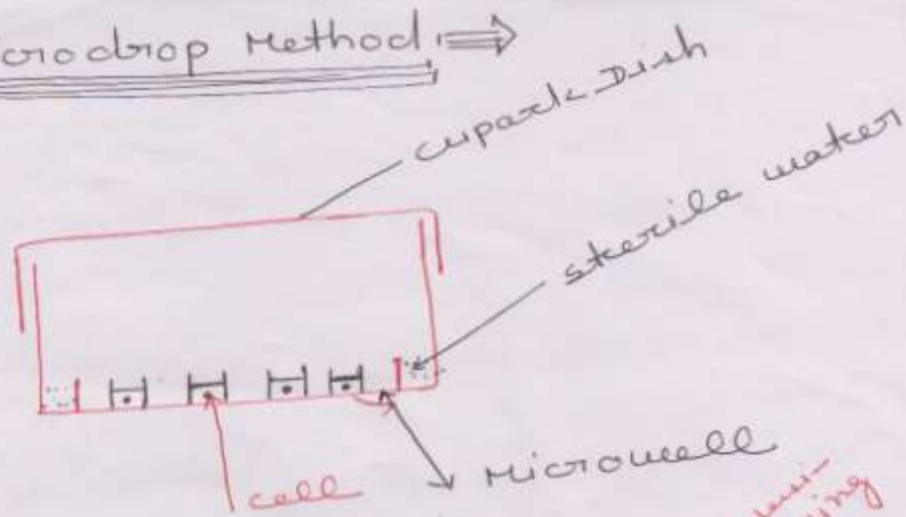
Microchamber Technique

(A microchamber can be created either by using a microscope slide and coverslips (the latter are held in place by sterile mineral oil) or by a cavity slide. Single cells are suspended in conditioned medium, and a drop of medium having a single cell is placed in the microchamber, which is covered with a coverslip). In case of cavity slide, the drop is placed onto a coverslip which is then inverted into the slide cavity.



Microchamber Technique

Microdrop Method ⟹

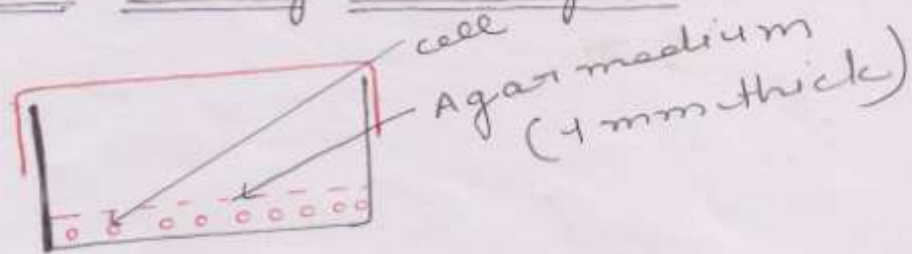


Microdrop technique

A specially designed dish, Cupark dish, having a smaller outer chamber (to be filled with sterile distilled water to avoid desiccation of cells) a larger inner chamber (having several numbered microwells) is employed. Microdrops of 0.25-0.5 μl are distributed in the microwells and the dish is sealed with parafilm. Cell density in the medium is so adjusted as to give, on an average, one cell per droplet (it works out as 2-4 × 10^3 cells/ml)

This method has been successfully used for protoplasts.

Bergman's Plating technique -



In this widely used technique the cells are suspended in a liquid medium. sterilized agar medium is kept melted in a water bath at 35°C. Equal volumes of the liquid medium containing cells and the agar medium are mixed thoroughly and quickly spread in 1mm thick layer in petridish. The cells remain embedded (embedded) in the soft agar medium and observable under a microscope; when macroscopic colonies develop they are isolated and cultured.

Thin layer liquid medium ⇒

cells can be plated in a thin layer of liquid medium to allow adequate aeration since cells are not fixed in position, This technique is common for protoplast culture.