PLANT ANATOMY

CONCEPTS AND CHALLENGES IN PROTOPLASTS ISOLATION AND CULTURE

Presented by

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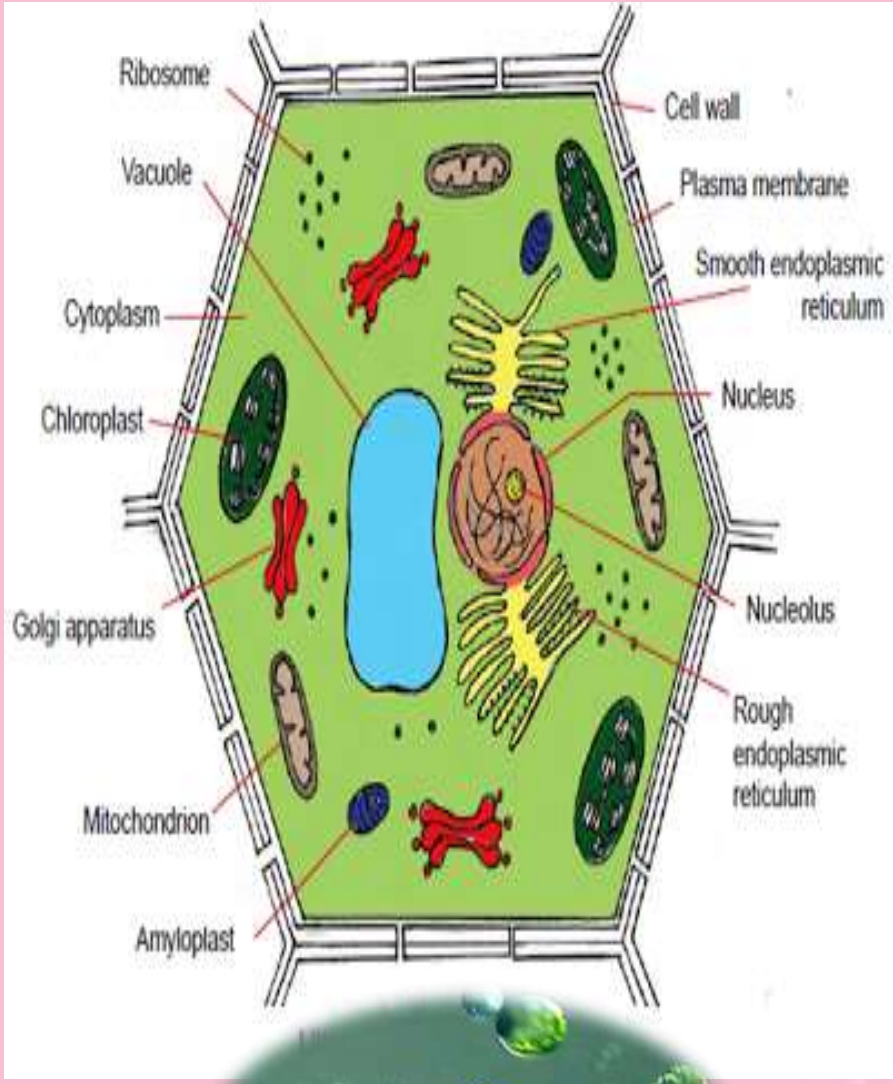
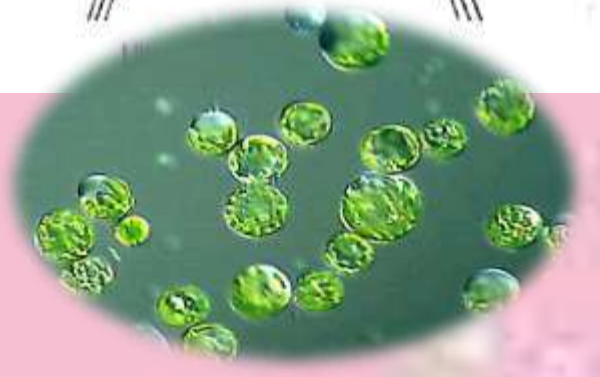
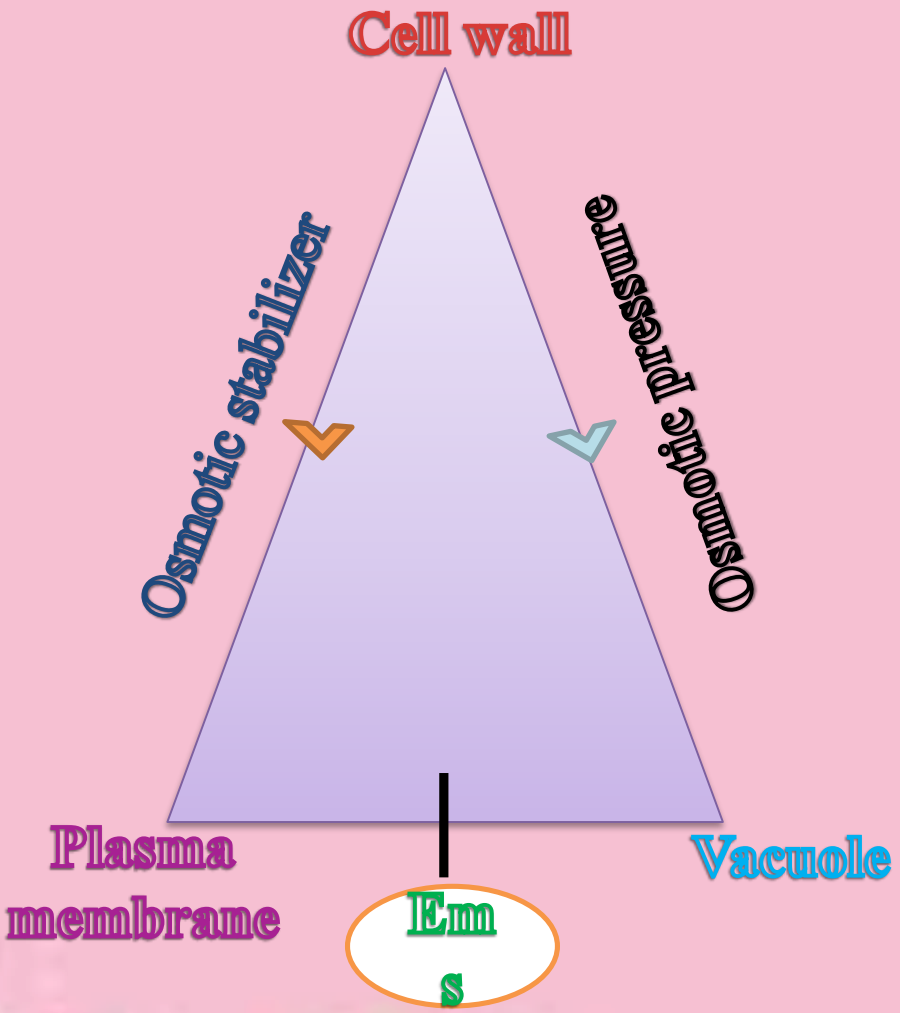
TRICHY – 24.


Ribosome
Vacuole
Cytoplasm
Chloroplast
Golgi apparatus
Mitochondrion
Amyloplast
Cell wall
Plasma membrane
Smooth endoplasmic reticulum
Nucleus
Nucleolus
Rough endoplasmic reticulum




Cell wall
Osmotic stabilizer
Osmotic pressure
Plasma membrane
Vacuole
Em
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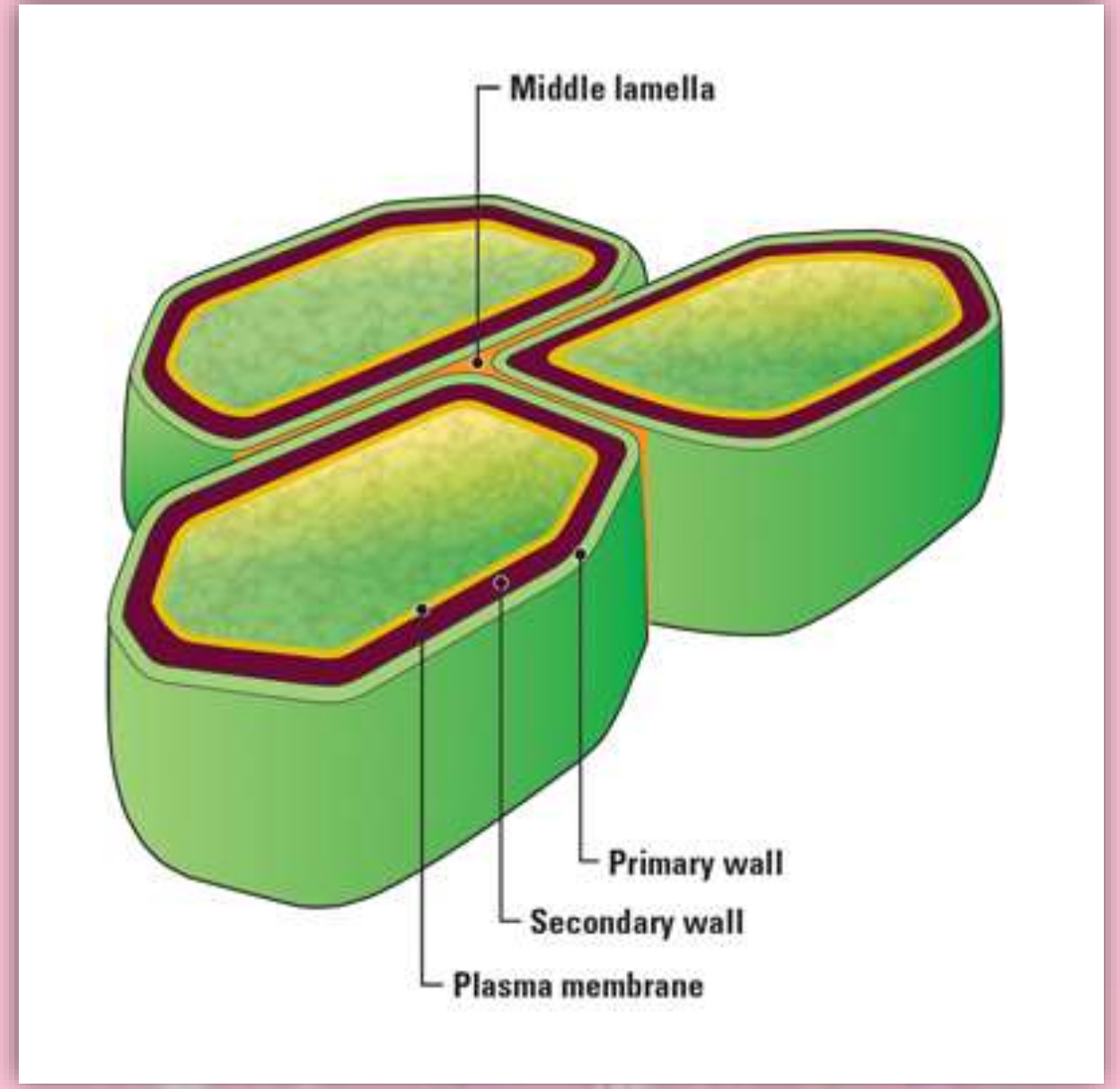

Middle lamella
Primary wall
Secondary wall
Plasma membrane

The Primary Cell Wall (composition and texture)

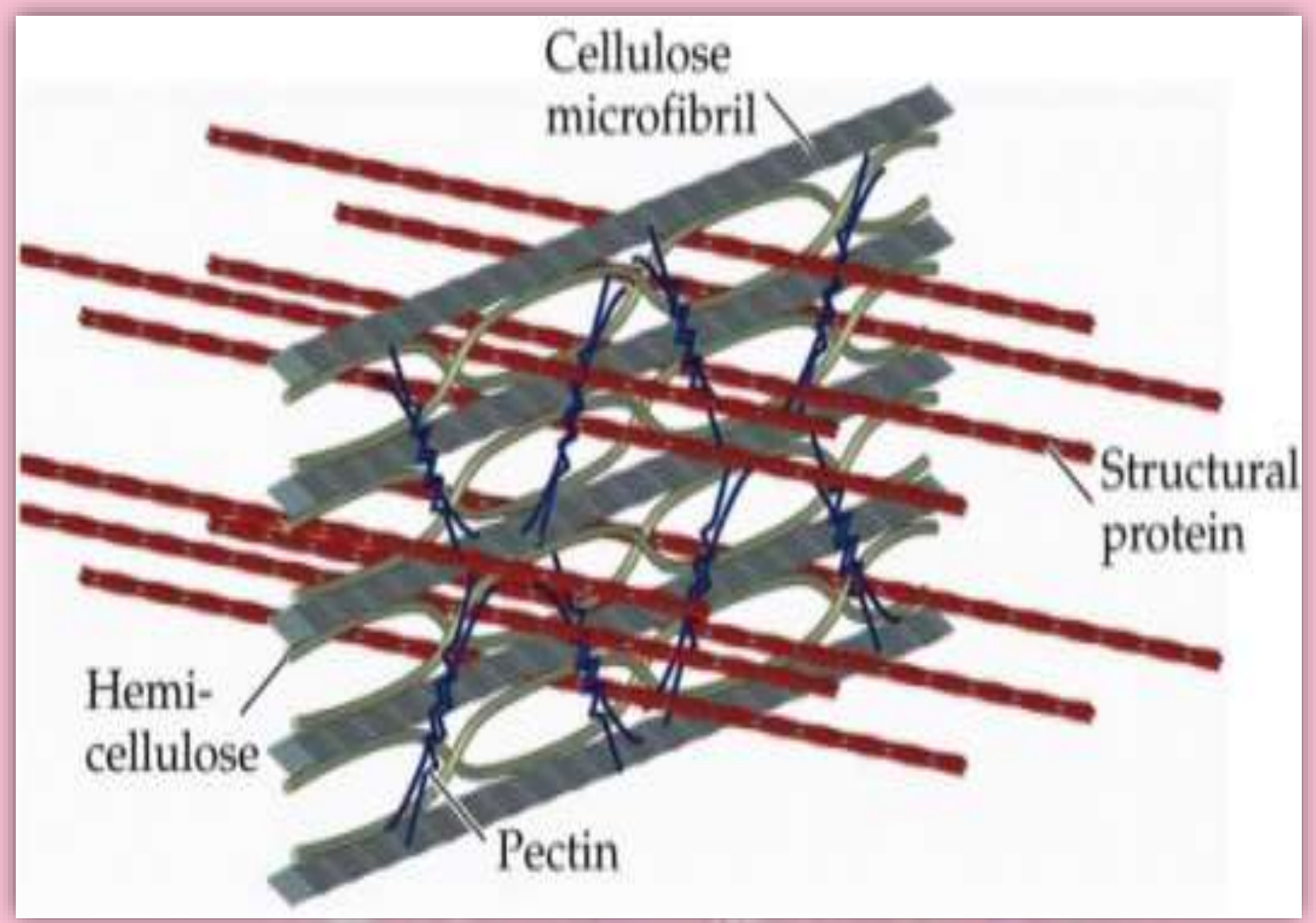
Three Major Polysaccharides

a) Cellulose:

- B 1-4 linkage, long unbranched linear chain of glucose, with crystalline properties because of arrangement of **cellulose**.
- Found in a form **Microfibrils are bundles** of about 30 thread-like cellulose molecules (10-25 nm in diameter) orderly arranged in parallel arrays forming an extended three-dimensional lattice characteristic of crystals **(called micelles).**
- **Macrofibrils** in secondary walls.

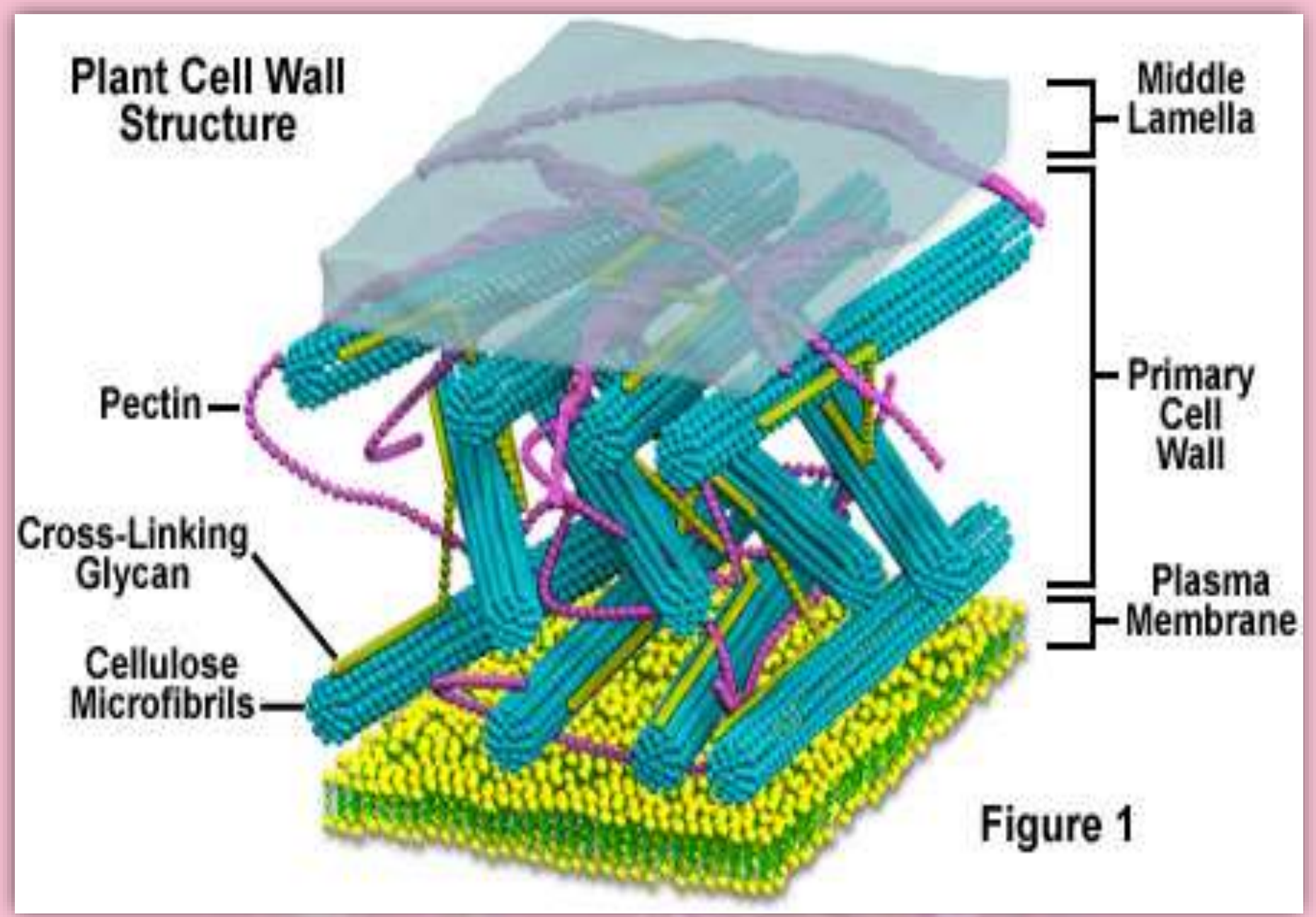
b) Hemicellulose:

- Highly branched long chains of glucose (xyloglucans, xylans)
- Microfibrilsare coated with the fibrous hemicellulose= xyloglucan.
- Hydrogen bonds with cellulose.
- Xyloglucanis, in turn, chemically bonded to another hemicellulose that serves as a cross-link between pectin molecules.


Cellulose microfibril
Structural protein
Hemi-cellulose
Pectin

c) **Pectins:**

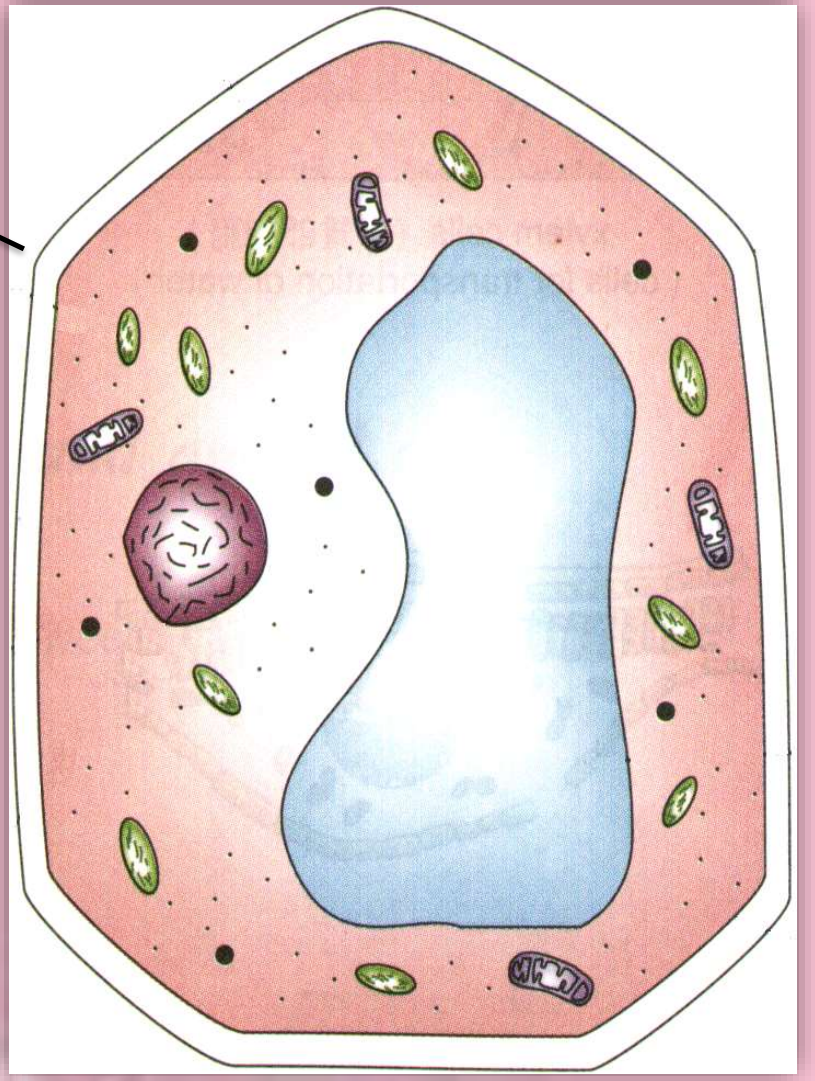
Cellulose and hemicellulose embedded in.-Form a separate network that interdigitates with the cellulose-hemicellulose network.-Note middle lamella (region between cells)is composed of pectin-glues cells together-Hydrophilic = holds up to 65% water in primary walls-function in Cell adhesion, regulate porosity.

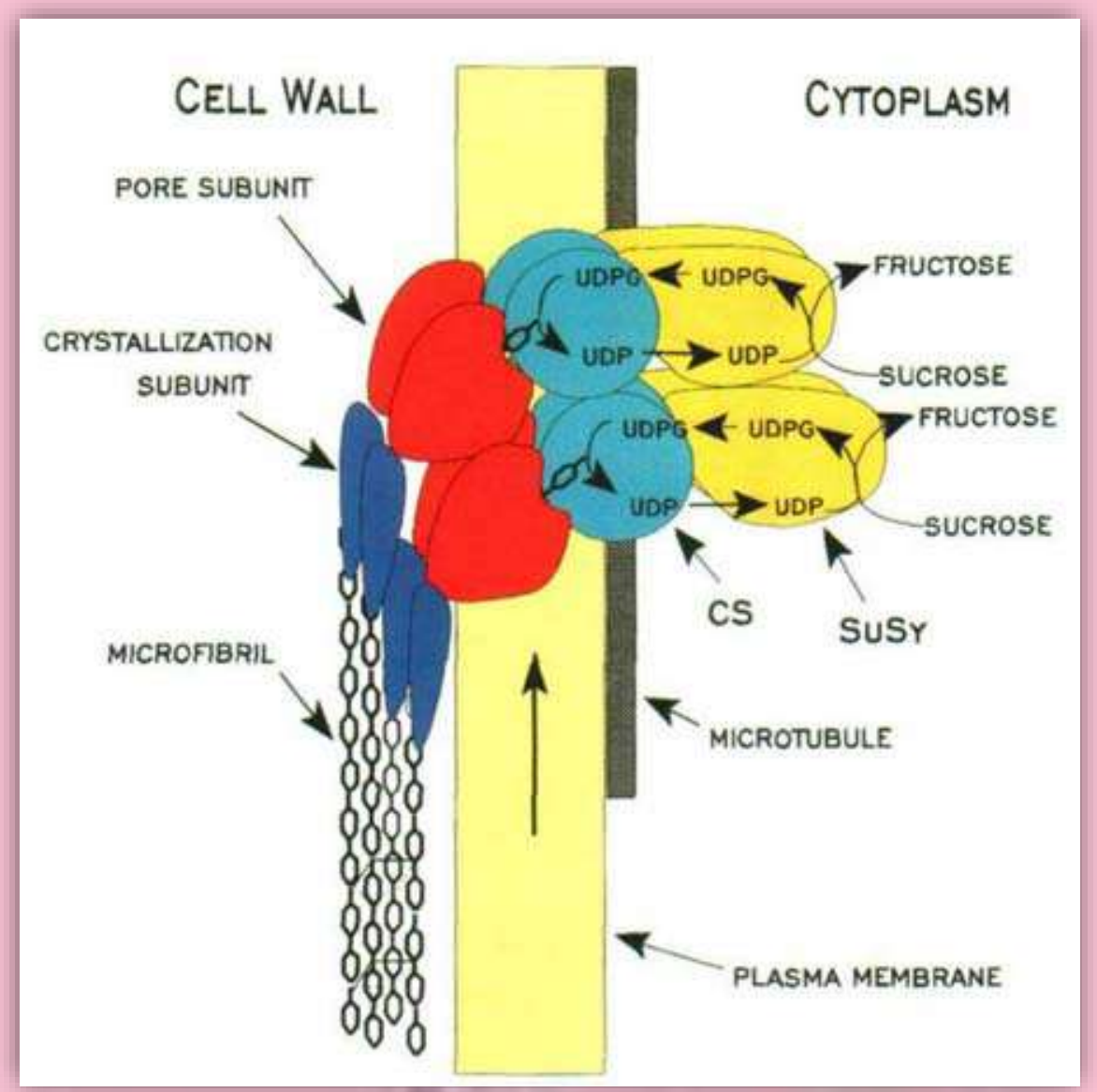

Plant Cell Wall Structure
Middle Lamella
Primary Cell Wall
Plasma Membrane
Pectin
Cross-Linking Glycan
Cellulose Microfibrils
Figure 1

Cell membrane

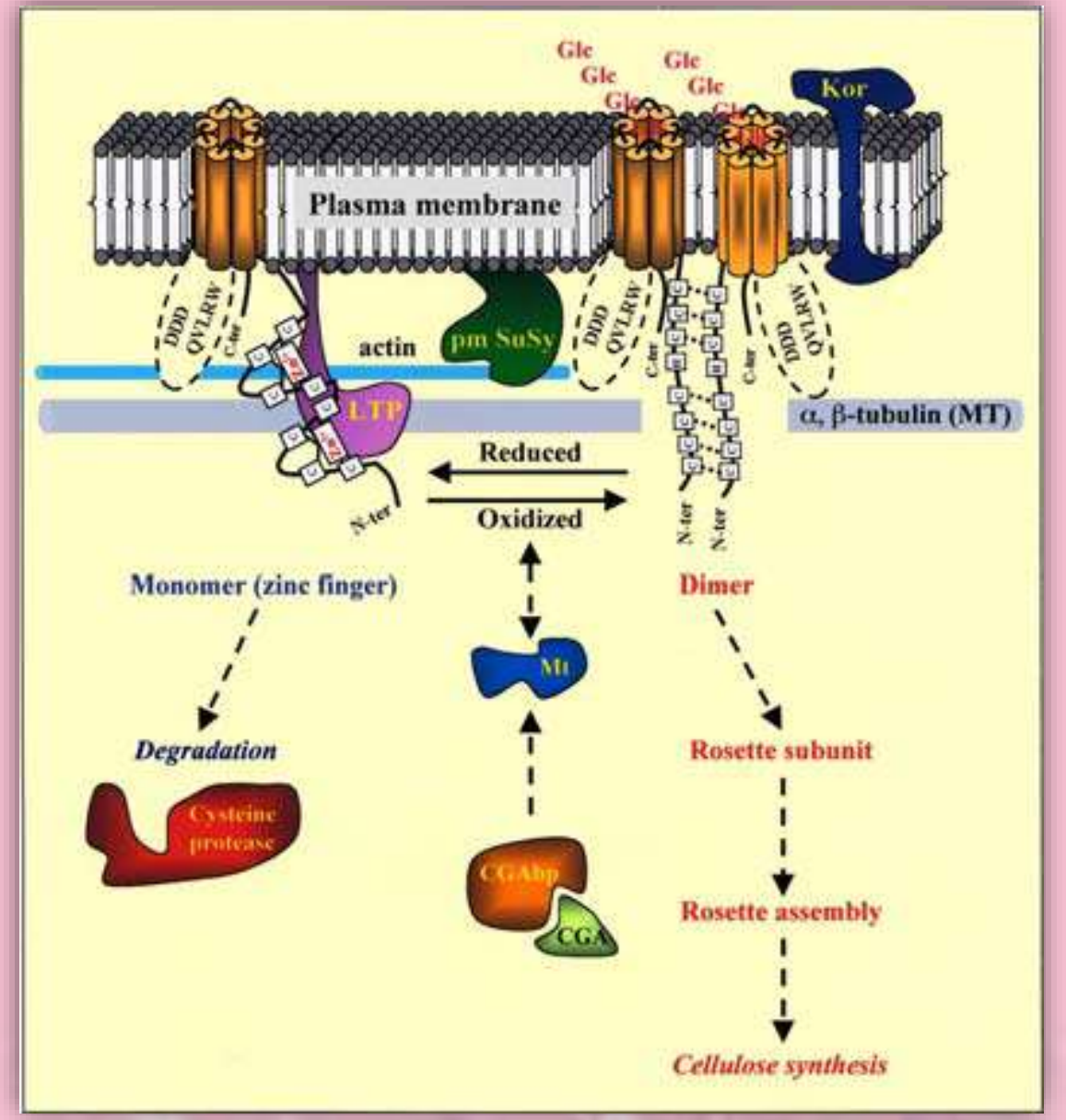
- Lies immediately against the cell wall
- Made of protein and lipid

  ∴ Selectively permeable





Delmer and Amor, 1995, Plant Cell 7:987

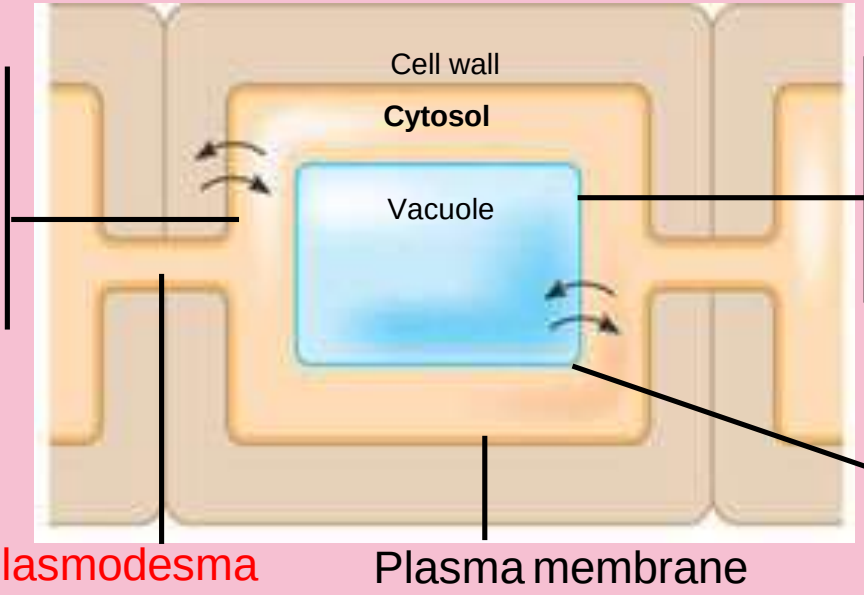


Doblin et al., 2002, Plant Cell Physiol. 43:1407

- The vacuole is a large organelle that can occupy as much as 90% of more of the protoplast's volume
- **The vacuolar membrane :**

  Regulates transport between the cytosol and the vacuole.

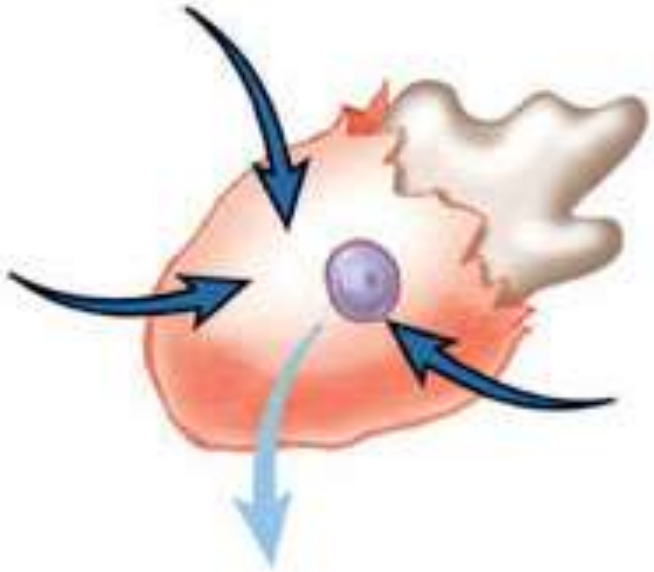
Transport proteins in the plasma membrane regulate traffic of molecules between the cytosol and the cell wall.



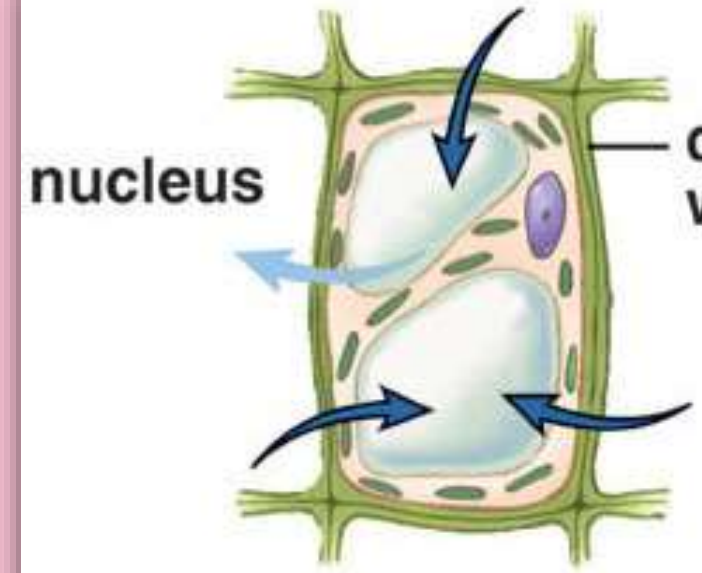
Transport proteins in the vacuolar membrane regulate traffic of molecules between the cytosol and the vacuole.

Cell compartments. The cell wall, cytosol, and vacuole are the three main compartments of most mature plant cells.

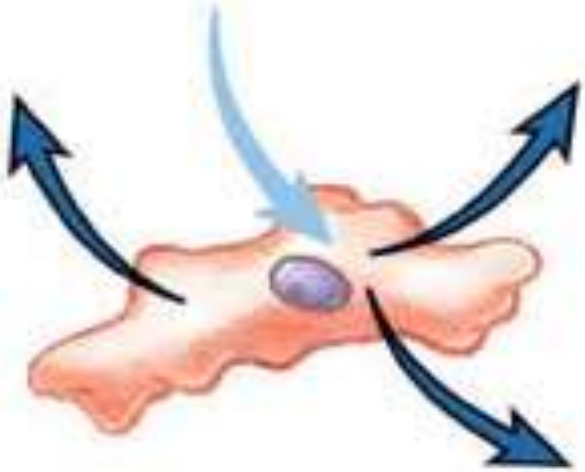
HYPOTONIC SOLUTION



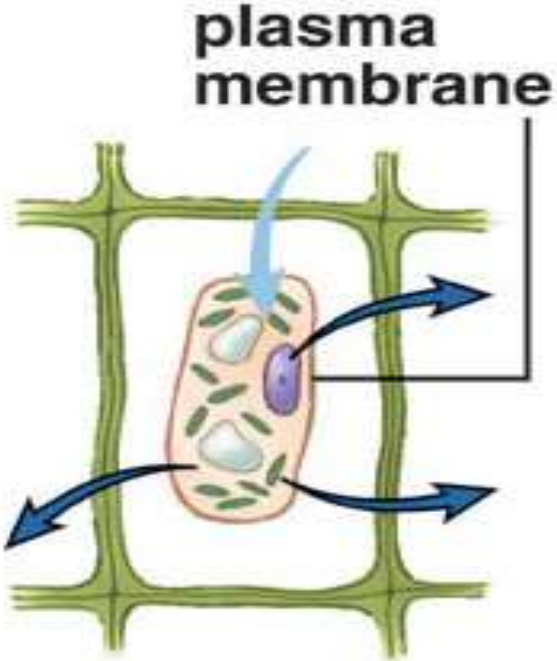
In a hypotonic solution, water enters the cell, which may burst (lysis).



In a hypotonic solution, vacuoles fill with water, turgor pressure develops, and chloroplasts are seen next to the cell wall.

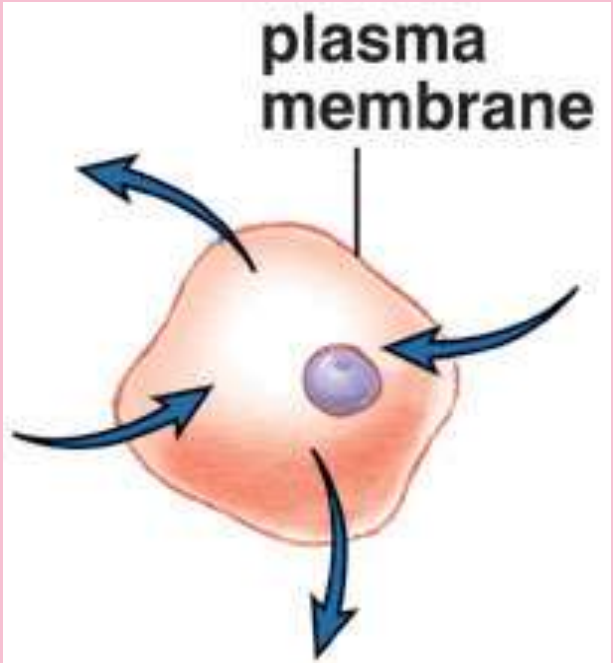


In a hypertonic solution, water leaves the cell, which shrivels (crenation).

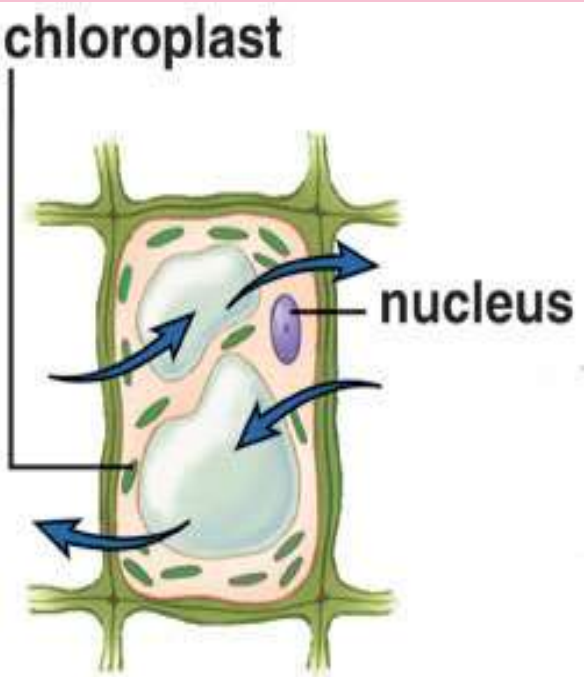


In a hypertonic solution, vacuoles lose water, the cytoplasm shrinks (plasmolysis), and chloroplasts are seen in the center of the cell.

ISOTONIC SOLUTION

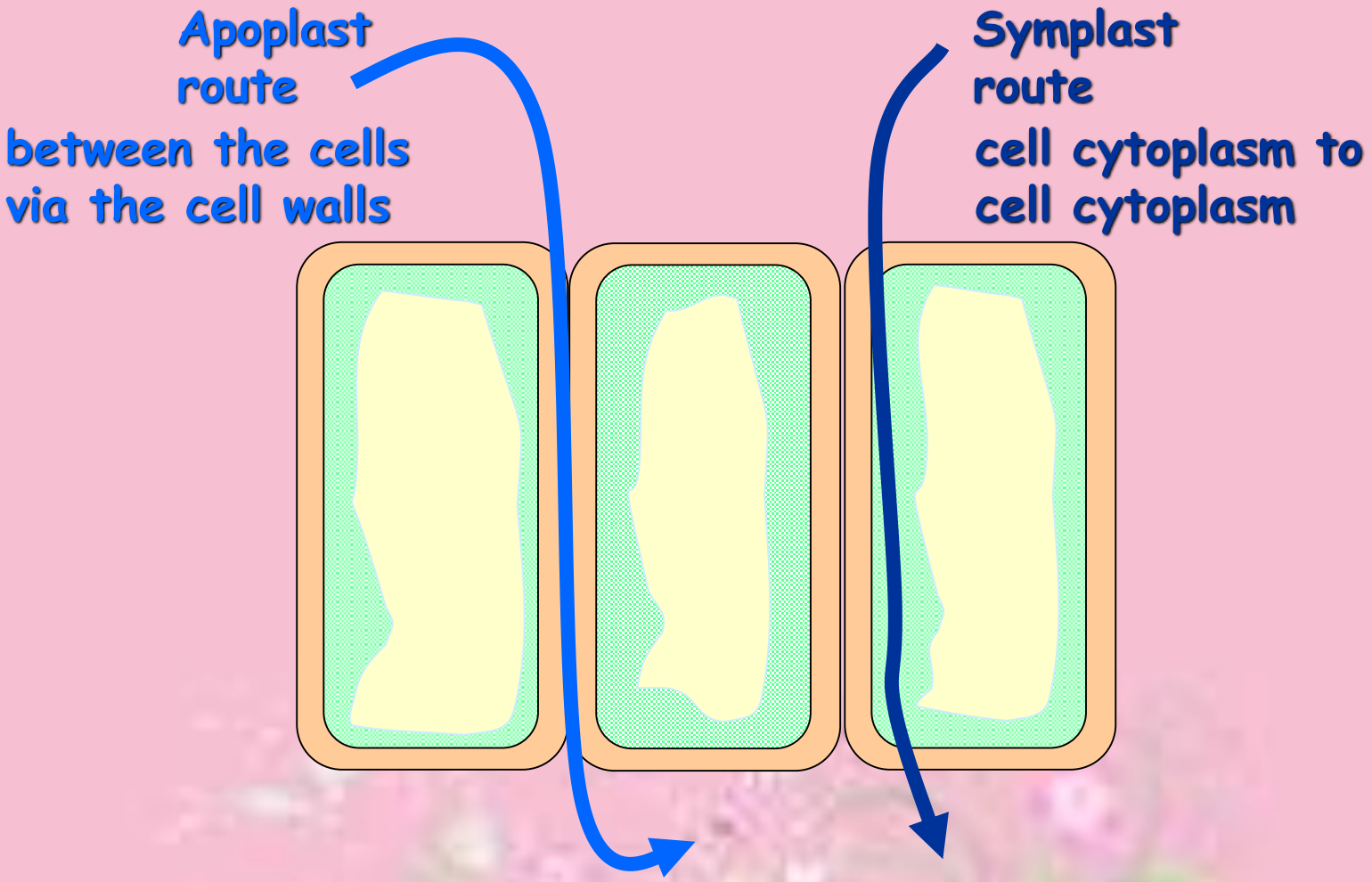


In an isotonic solution, there is no net movement of water.

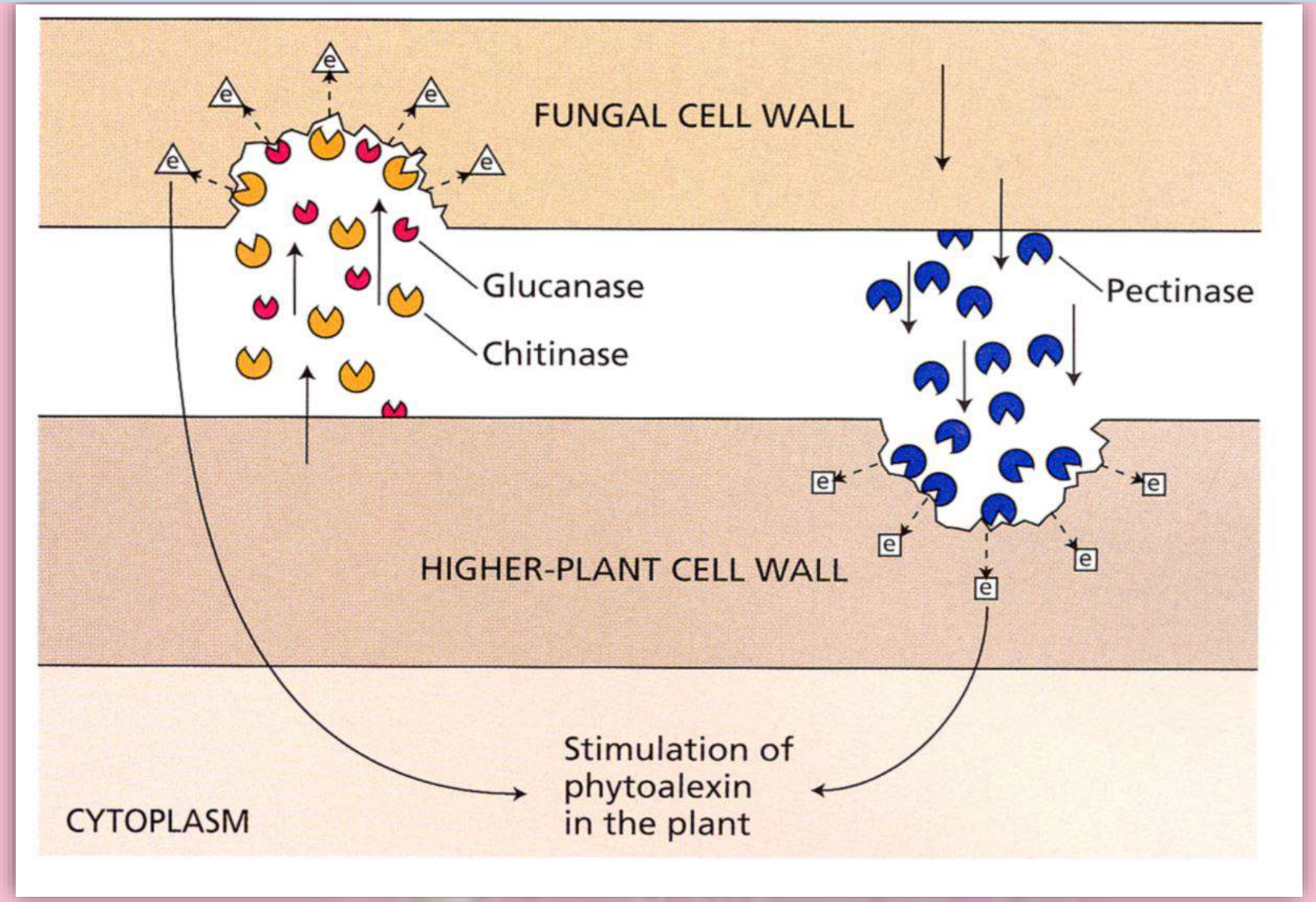


In an isotonic solution, there is no net movement of water.

Water is transported across the root by two routes

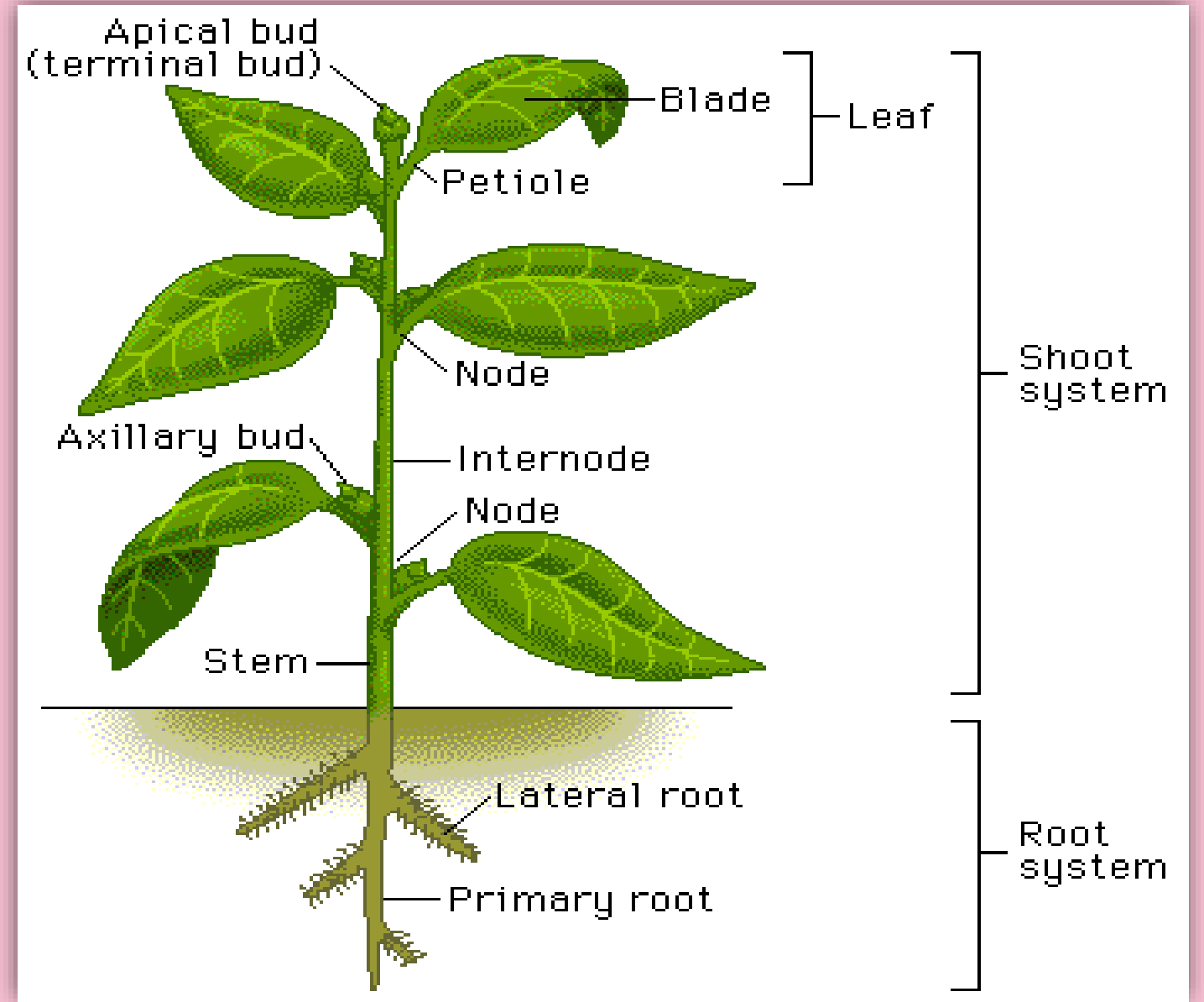


Plant pathogens secrete cell-wall-degrading enzymes



PROTOPLASTS

- Naked plant cells, all components of a plant cell excluding the cell wall.
- Cell wall degrading enzymes (**Cellulase**).
- Used in cell fusion studies, and to take up foreign DNA, cell organelles, bacteria and virus particles.


Apical bud
(terminal bud)
Blade
Leaf
Petiole
Node
Axillary bud
Internode
Node
Stem
Shoot
system
Lateral root
Primary root
Root
system

Source of explant tissue

- Explants tissue should be young and contain a high proportion of meristematic cells.
- The tissue consists of cells without excessive secondary thickening of the cell walls.
- Source tissue in dicots is provided by young expanding leaves which must be surface-sterilized without damaging the tissue.
- A continuous supply of surface-sterile source tissue can be obtained by subculture of shoot tips of Dicots.
- It is difficult to regenerate plants from leaf tissue of monocots
- Immature embryos excised from fertilized flowers produces embryogenic callus, which provides source tissue for protoplast isolation.

Protoplast isolation

1. Mechanical method :

•Suitable for large and highly vacuolated cells, such as onion bulb scales cells are plasmolysed, tissue is dissected and deplasmolyzed .

2. Enzymatic method :

• Commercial mixture of cell wall degrading enzymes in solution containing **osmotic stabilizers.**

Enzymatic protoplast isolation

- Protoplasts have been isolated from a variety of tissues and organs including **leaves, petioles, shoot apices, fruits, roots, coleoptiles, hypocotyls, stem, embryo, microspores, callus and cell suspension cultures** of a large number of plant species.

- Most suitable source is mesophyll tissue from fully expanded leaves of young plants or new shoots: large number of uniform cells, loosely arranged.

ENZYMES

Pectinase mainly degrades the middle lamella **Cellulase** and **hemicellulase** are required for other main component Helicase, colonase, cellulysin, glusulase, zymolyase, pectolyase etc.

1. Two-step or sequential method

   Pectinase (or macerozyme) → **Cellulase**

2. One-step or simultaneous method:

   one mixture of enzymes

Osmoticum

- In isolating protoplasts, the wall pressure that is mechanically supported by cell wall must be replaced with an appropriate osmotic pressure (also later in the culture medium).
- Osmotic stress has harmful effects on cell metabolism and growth: condensation of DNA in cell nuclei and decreased protein synthesis.
- Lower osmotic potentials by addition of various ionic and non-ionic solutes: ***mannitol***, **sorbitol, glucose, fructose, galactose, NaCl, CaCl etc**.

PROTOPLASTS ISOLATION PROCEDURE

- Where the source tissue is leaves, stems or roots, it is cut into narrow strips and is maintained for one hour in the osmoticum solution which contains inorganic salt mixture such as CPW salts and 13% w/v mannitol as an osmoprotectant to prevent the cell from losing or gaining water after wall removal.
- If the source tissue is a tissue culture, the cells are broken up into smaller aggregates then maintained in the osmoticum at 25^{o}C
- The tissues are then exposed to the osmoticum, for example **CPW plus 13% mannitol** containing the lytic enzymes, either as a sequential or a mixed enzymes treatment

- The period of exposure depends on the source tissue and on the strength of the enzymes, for example, the sliced leaves of most species can be incubated in 1% cellulysin, 0.1% macerozyme R10 and CPW13M medium at pH 5.6 overnight in the dark.
- For cereals, where the leaves have been stripped of the epidermis, the mixture consists of 2% cellulysin, 0.2% mazerozyme R10, 0.5% hemicellulase, 1% potassium dextran sulphate, 11% mannitol at pH 5.8 using a Tris-malate buffer and incubated in the dark for 1-2 h and for most tissue cultures the incubation mixture consists of 2% rhozyme HP150, 2% meicelase, 0.03% macerozyme R10 and CPW13M medium at pH 5.8 and incubated in the dark overnight

- The cells are then freed of this mixture by filtering through a fine mesh filter (64 mM mesh size) and centrifuging at low speed (100g for 10 min)
- The protoplasts are resuspended in 5 ml osmoticum without the lytic enzymes then the suspension is centrifuged as before
- The pellet is separated from the debris by transferring by pipette to a dense solution (19-20% sucrose or 30% Percoll) then the centrifuging repeated which causes the debris to sink and the protoplasts to rise to the surface.
- The protoplast band is then moved carefully by a pipette and transferred to a culture medium (MS medium, 2.0 mgl^{-1}NAA, 0.5 mgl^{-1} BAP, 3% sucrose and 9% mannitol)

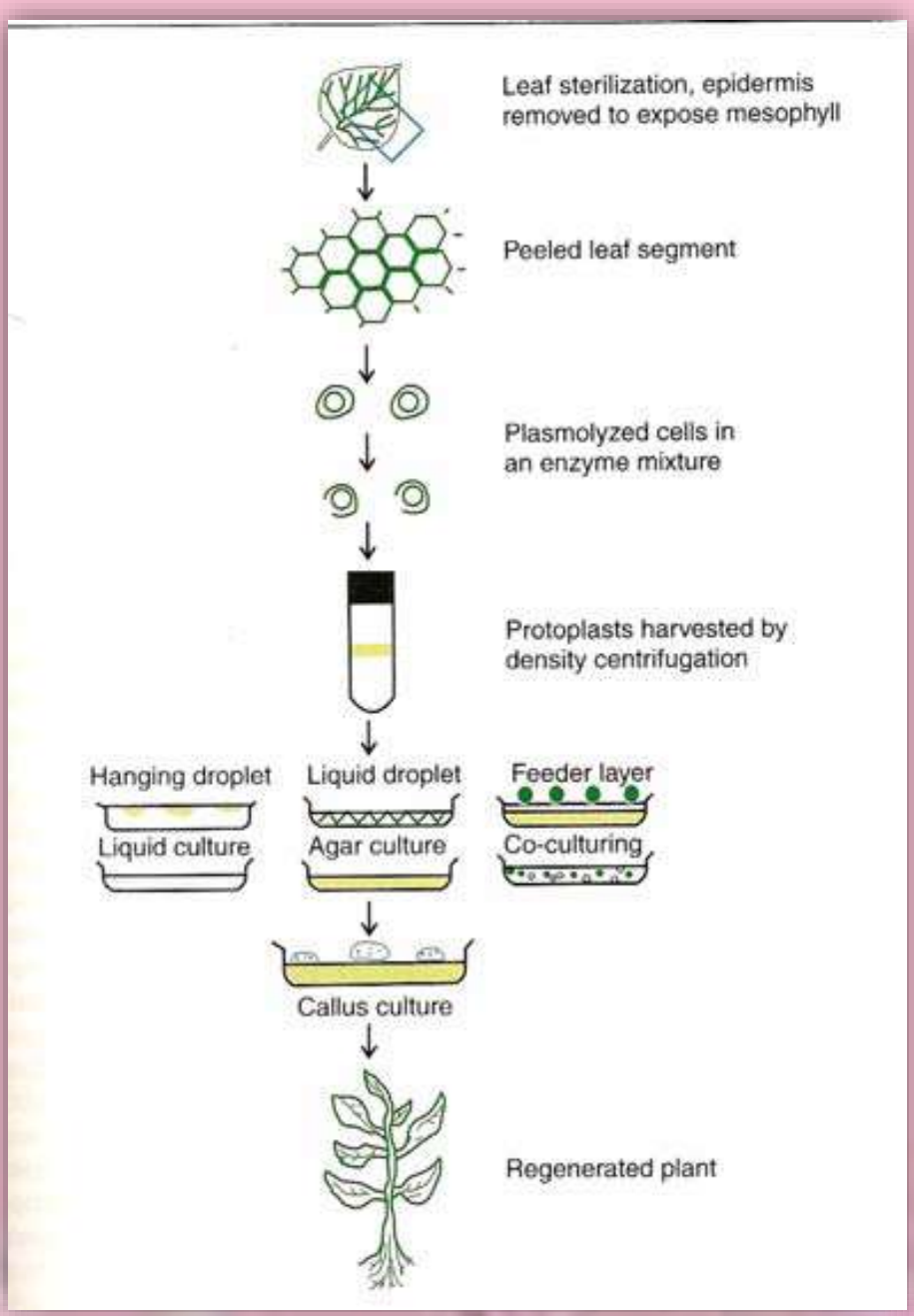
Suspension cell-derived protoplasts of rye (*Secale cereale* L.)

Enzyme mixture	Composition[a]	Yield of purified protoplasts ($\times 10^6$ g^{-1} FW)[b]	Viability (%)
Solution 1	3% Cellulase Onozuka RS (Sigma), 0.2% pectolyase Y23 (Sigma), 1% driselase, 1% macerozyme R10 (Sigma), 1% $CaCl_2.H_2O$, 0.1% $MgSO_4.7H_2O$, 0.05% KH_2PO4. pH 5.6	11.7a	90
Solution 2	3% Cellulase Onozuka RS (Sigma), 0.2% pectolyase Y23 (Sigma), 1% driselase, 1% $CaCl_2.H_2O$, 0.1% $MgSO_4.7H_2O$, 0.05% KH_2PO4. pH 5.6	8.9b	91
Solution 3	3% Cellulase Onozuka RS (Sigma), 0.2% pectolyaseY23 (Sigma), 1% macerozyme R10 (Sigma), 1% $CaCl_2.H_2O$, 0.5% hemicellulase, 0.1% $MgSO_4.7H_2O$, 0.05% KH_2PO4. pH 5.6	3.5c	92
Solution 4	3% Cellulase Onozuka RS (Sigma), 0.2% pectolyase Y23 (Sigma), 1% $CaCl_2.H_2O$, 0.1% $MgSO_4.7H_2O$, 0.05% KH_2PO4. pH 5.6	2.2c	91

Influence of culture density on the frequency of protoplast division and plating efficiency. Data are the means of five replicates

Culture density ($\times 10^6$ protoplasts/ml)	Division frequency[a] (%)		Plating efficiency[a] (%)	
	Liquid	Agarose	Liquid	Agarose
0.2×10^6	7.3d	6.5d	0.2f	0.15e
0.4×10^6	7.9d	8.6d	0.5e	0.6d
0.6×10^6	11.2c	12.9c	0.8d	1.0c
0.8×10^6	18.4b	19.5b	1.5b	1.4b
1.0×10^6	21.8a	23.8a	1.7a	1.9a
2.0×10^6	19.0b	18.2b	1.6ab	1.5b
5.0×10^6	12.0c	11.7c	1.2c	1.1c

Influence of culture density on the frequency of protoplast division and plating efficiency. Data are the means of five replicates


Leaf sterilization, epidermis removed to expose mesophyll
Peeled leaf segment
Plasmolyzed cells in an enzyme mixture
Protoplasts harvested by density centrifugation
Hanging droplet
Liquid culture
Liquid droplet
Agar culture
Feeder layer
Co-culturing
Callus culture
Regenerated plant

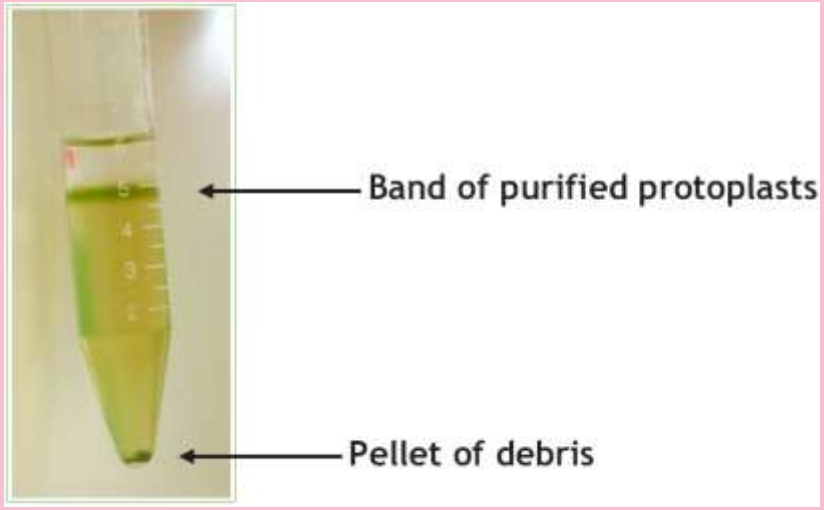
Protoplast Viability and Density

- FDA (fluorescein diacetate) staining accumulates inside the plasmalemma of viable protoplasts.

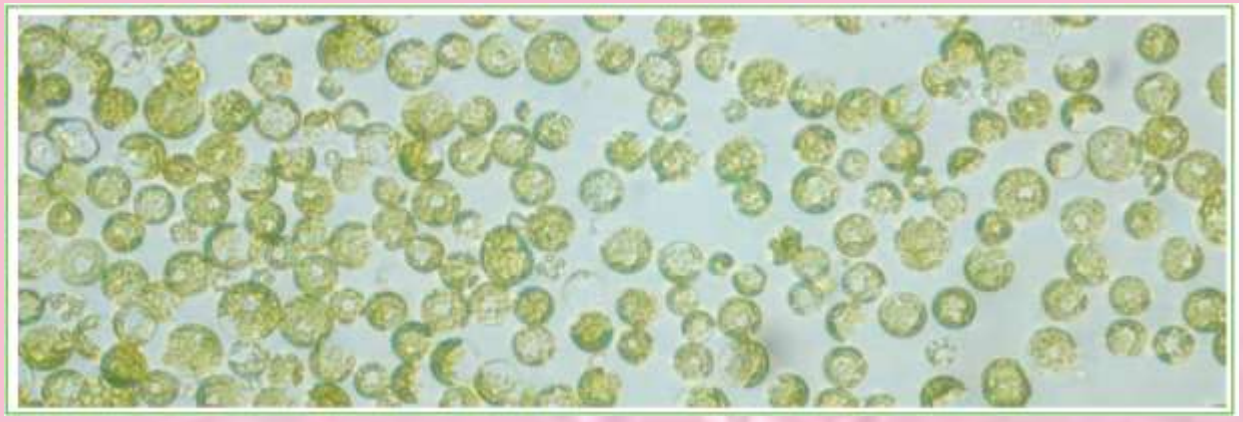
- Phenosafranine staining, Calcofluor White (CFW), oxygen uptake, photosynthesis.

- Maximum and minimum plating densities for growth.

Leaves were cut in thin stripes and digested in an enzyme solution with mannitol

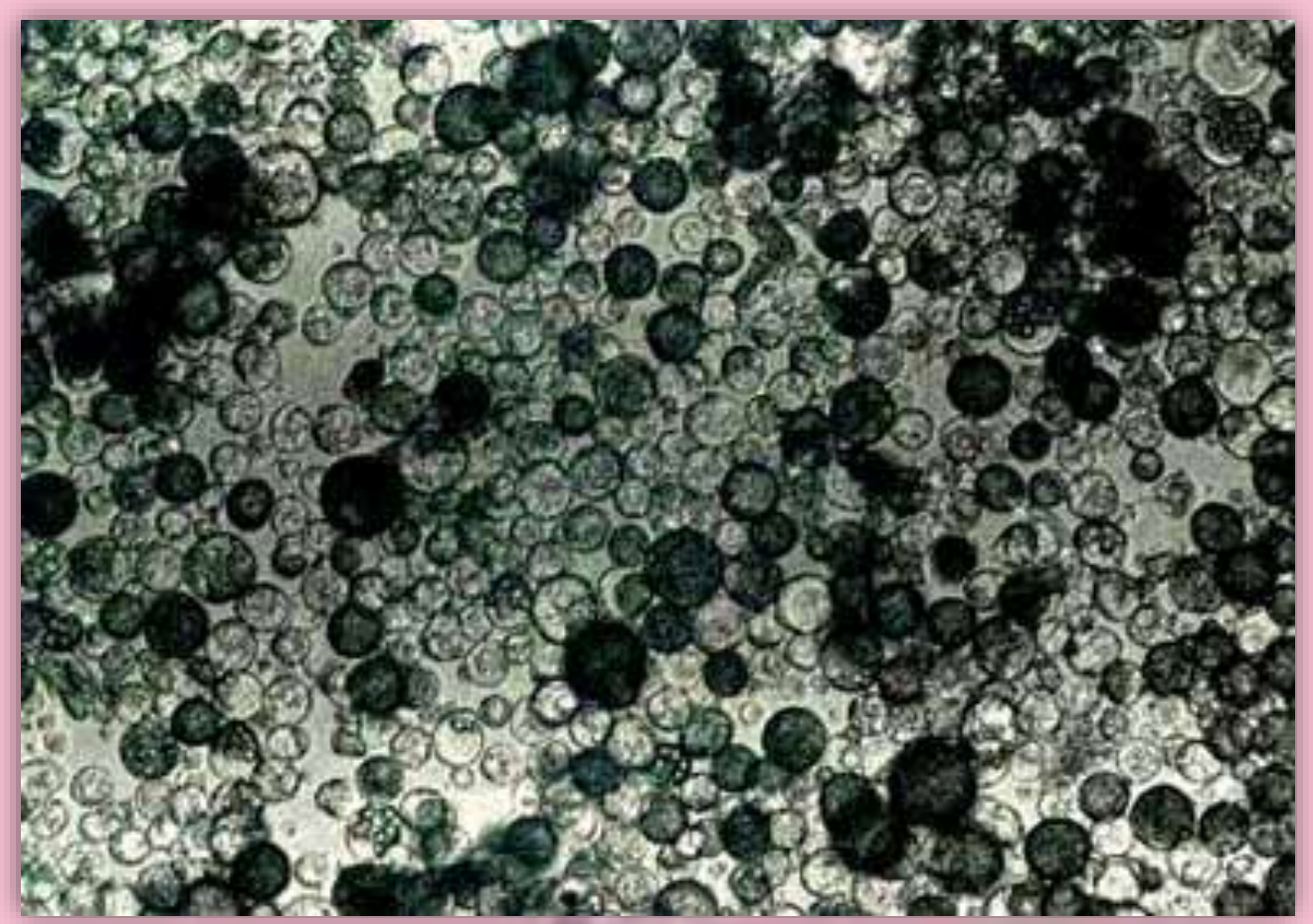


Purification of protoplasts from leaf debris on sucrose gradient



Purified protoplasts

Figure 1 Banana protoplasts



Protoplast culture techniques

1. Semisolid agar media:

- Protoplasts remain in a fixed position (no clumping)

2. Liquid culture:

- Easy dilution, transfer of protoplasts and adjustment of osmotic pressure

3. Liquid droplet method
4. Hanging droplet methods
5. Feeder layer
6. Co-culturing slow and fast growing protoplasts

Culture of isolated protoplasts

a. A typical medium would be MS medium 2.0 mgl^{-1} NAA, 0.5 mgl^{-1} BAP, 3% sucrose and 9% mannitol.

b. The experimental approach to establish the correct auxin and cytokinin levels required to stimulate growth of the protoplasts is based on a Latin Square arrangement.

c. A method of stimulating cell division and regeneration that is technically easier is to transfer the protoplast directly to a liquid or semi-liquid medium in which the levels of auxin and cytokinin are varied.

d. Normal agar is toxic to protoplasts, so it is replaced by low temperature gelling agaroses such as Sea Plaque, or Sigma.

Protoplast culture and fertile green plant regeneration of rye.

a) Friable embryogenic calli,

b) Suspension cell aggregates,

c) Freshly isolated protoplasts from suspension culture,

d) Protoplasts stained with FDA,

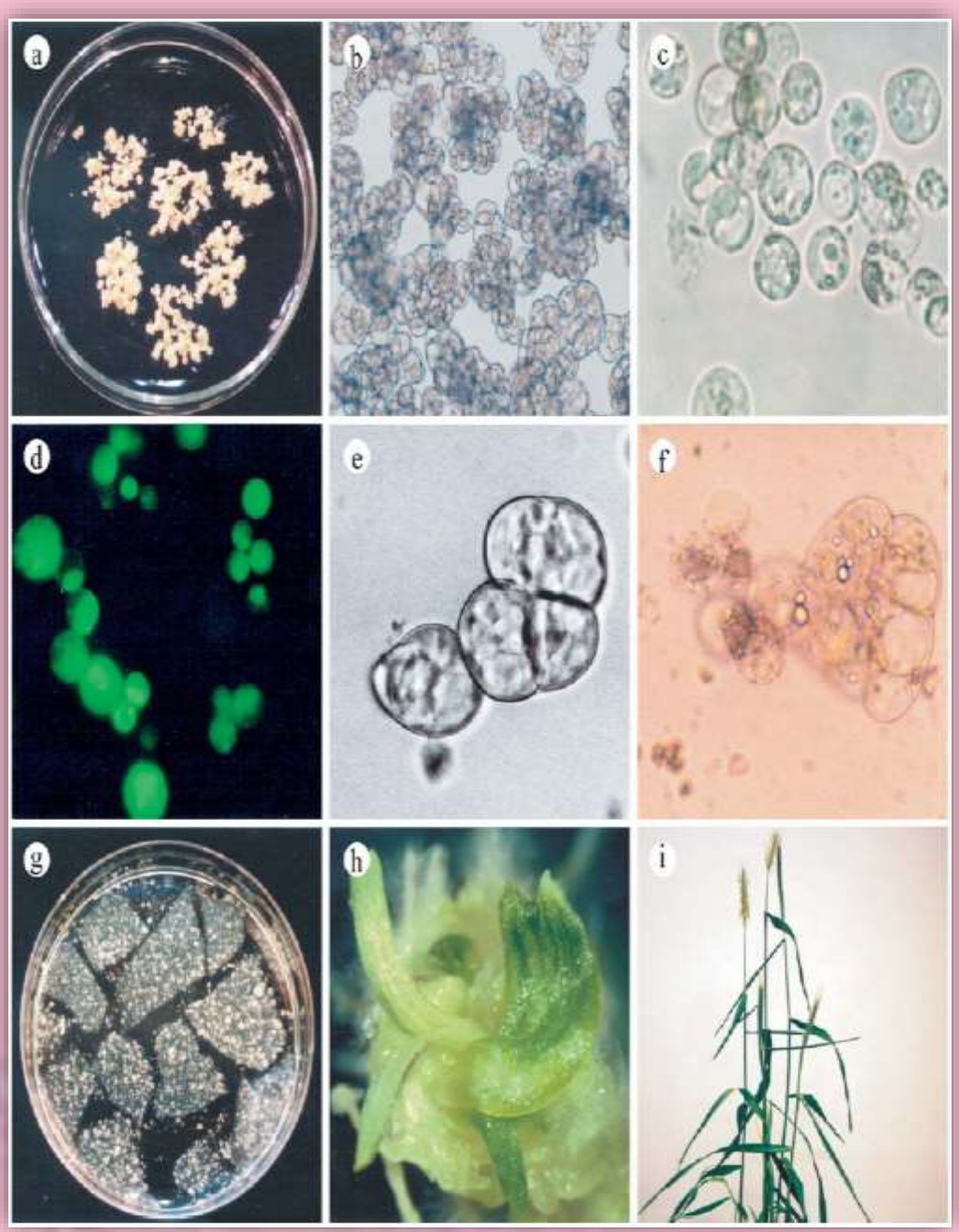
e) Division of cell derived from protoplasts,

f) Ell colonies derived from protoplast,

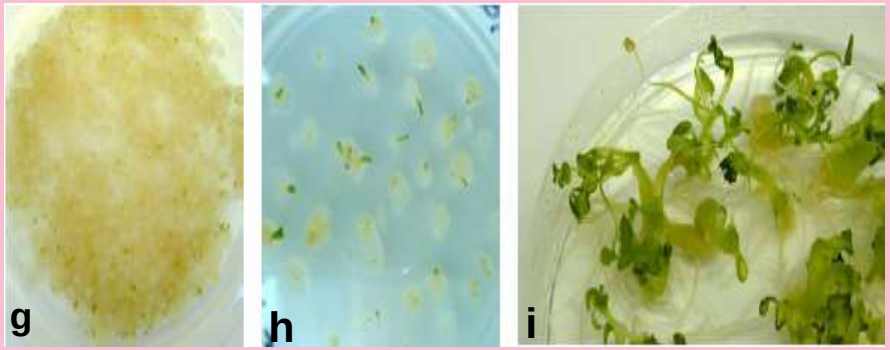
g) Proto-calli developed from protoplasts embedded in agarose,

h) Green shoot formation,

i) Fertile green plant.



Recovery of plantlets through somatic embryogenesis



a) First division, 10 days after protoplast isolation

b,c) 2 and 3 wo microcolonies.

d,e) 4 and 6 wo microcalli

f) 8 wo microcalli just before being transferred onto b5h medium

g) Transfer of microcalli onto B5H medium and induction of somatic embryos.

h) Transfer of globular embryos onto Boi2y medium for maturation of somatic embryos.

i) Transfer of cotyledonary stage embryos onto MS medium for conversion of embryo into plantlets.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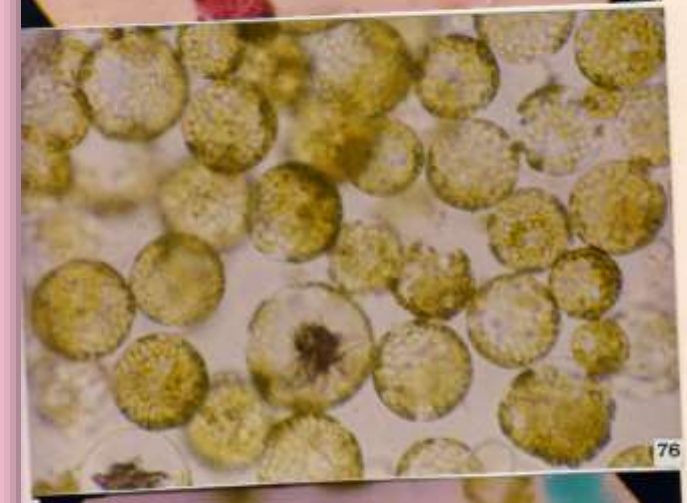
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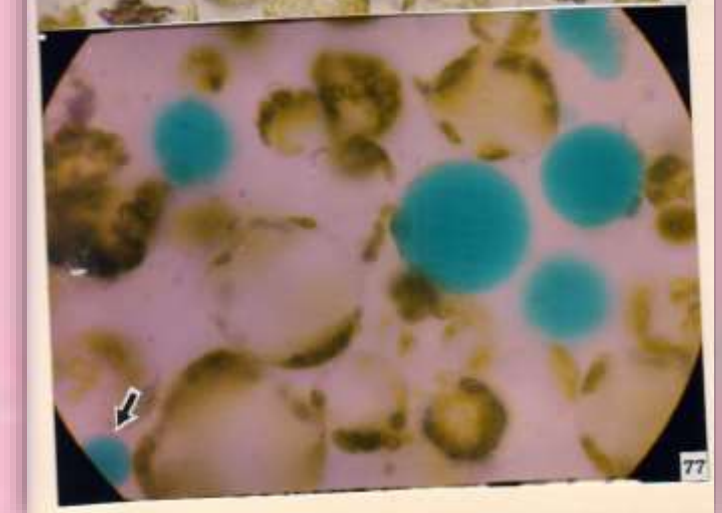
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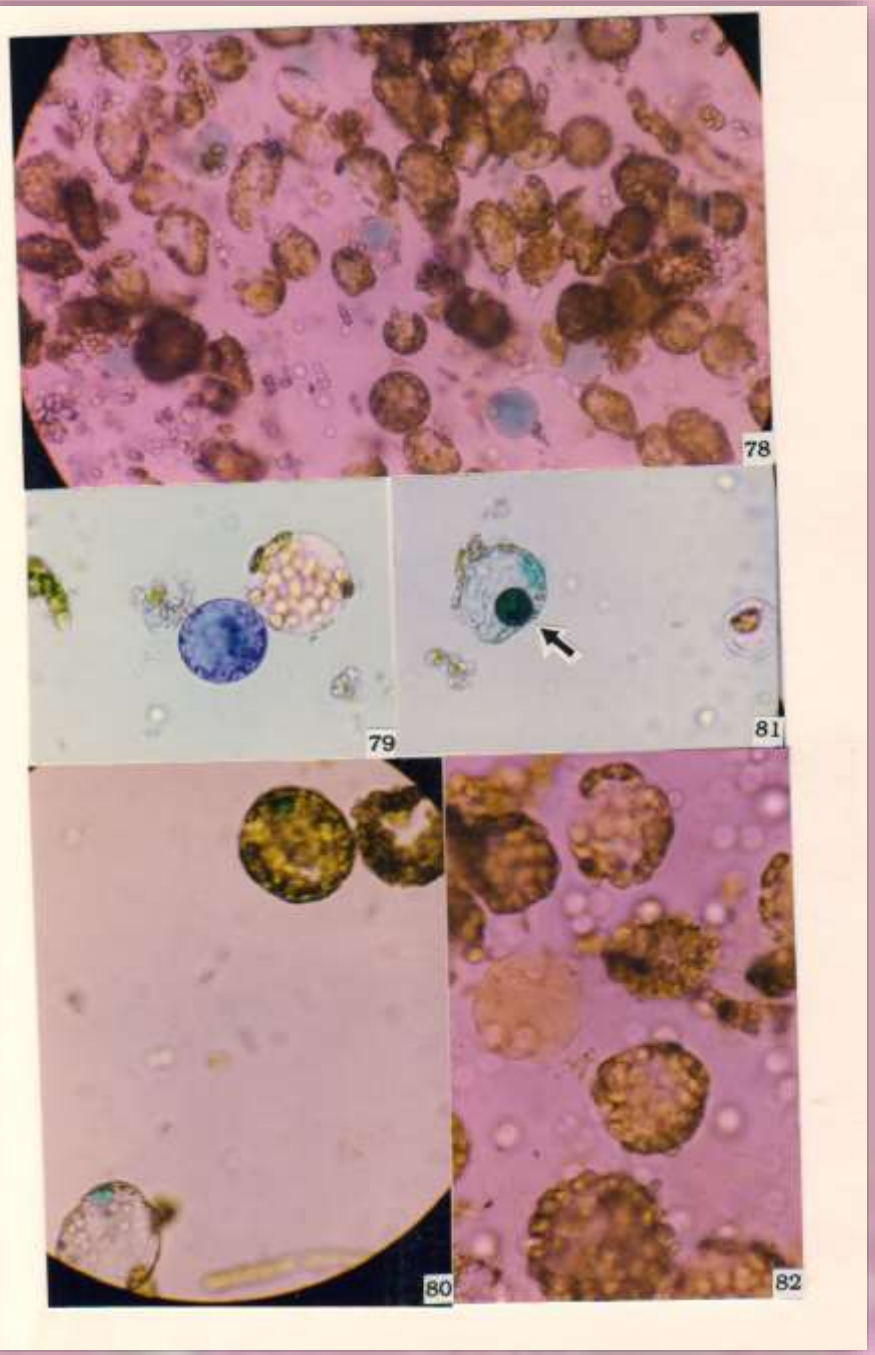
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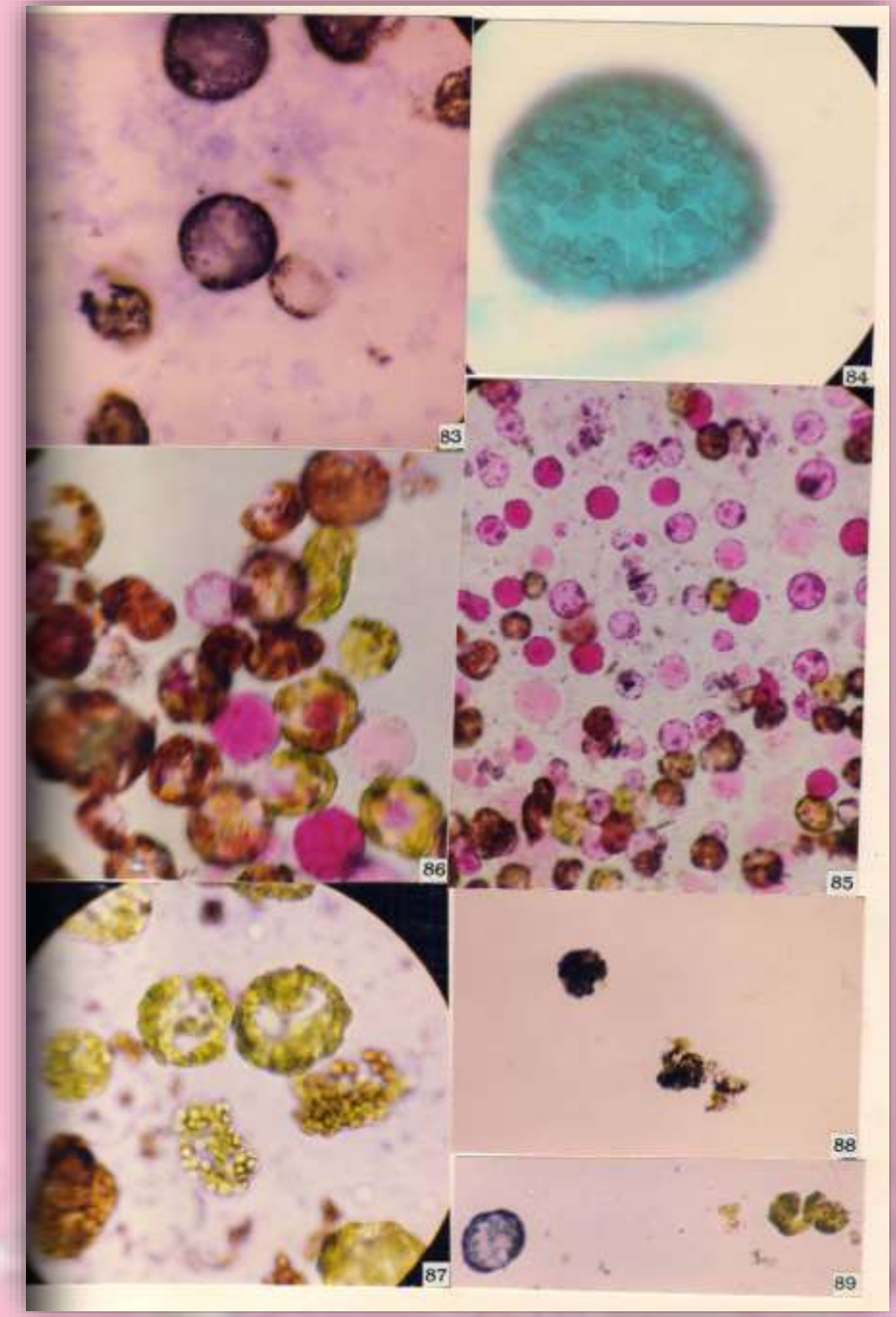
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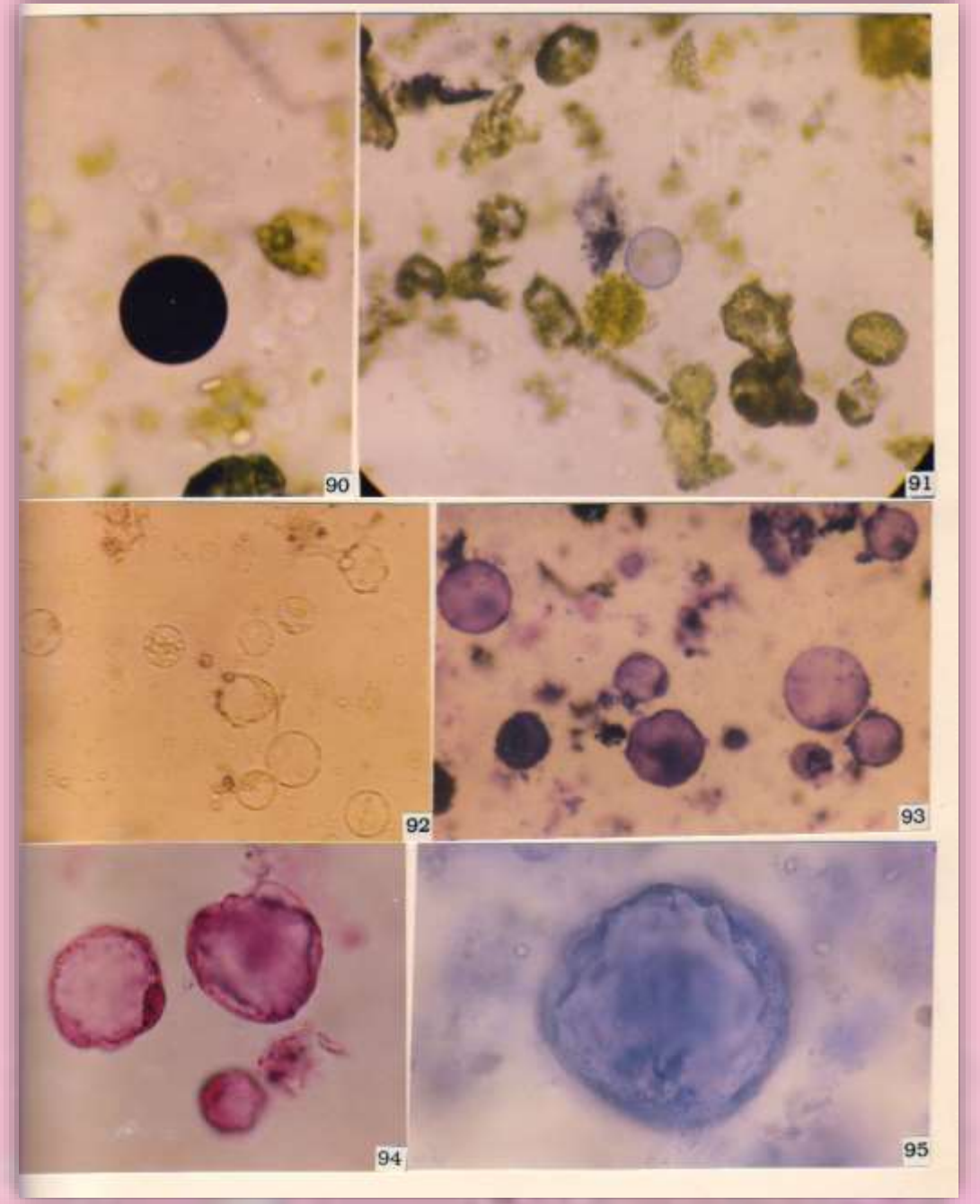
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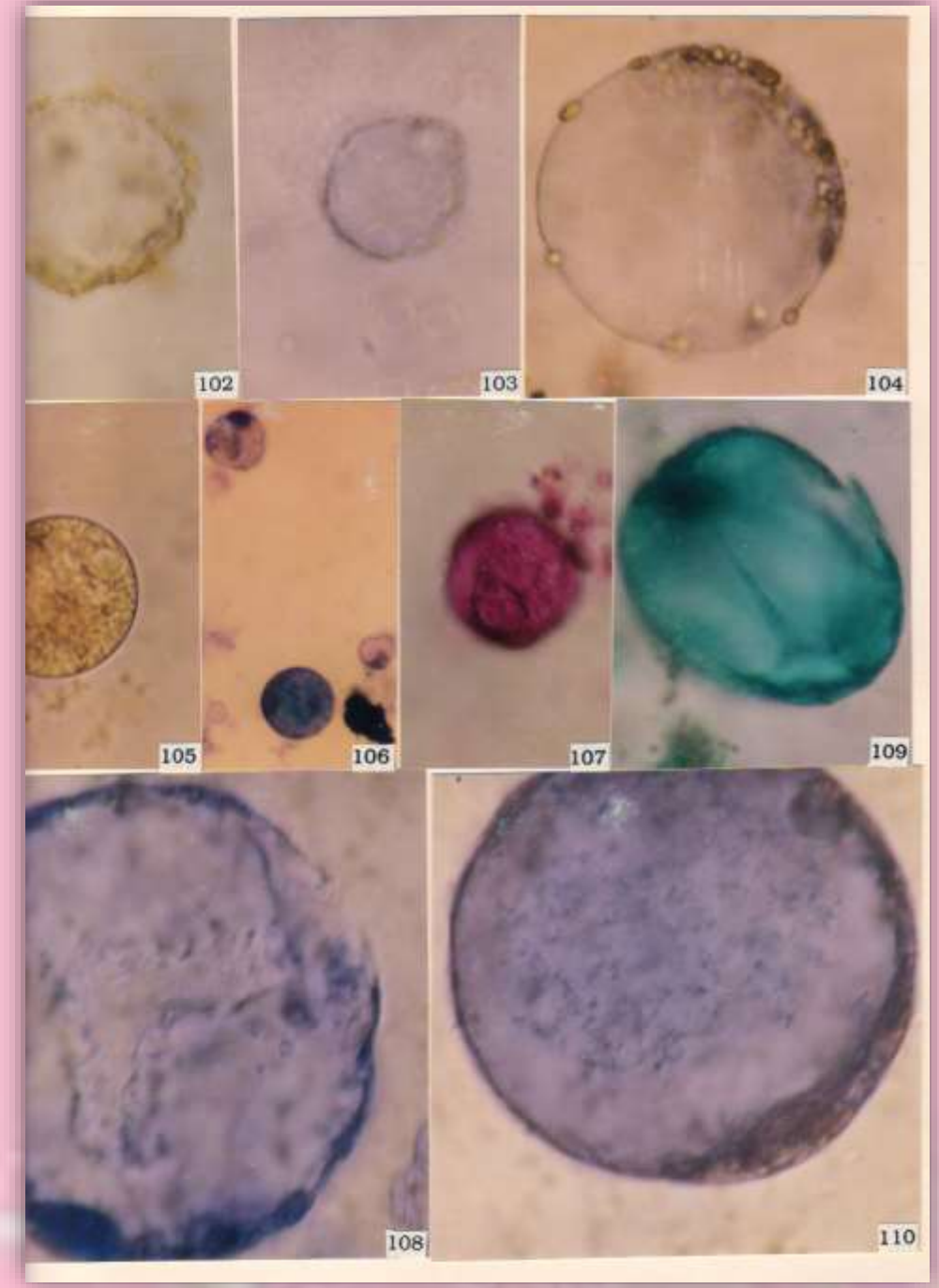
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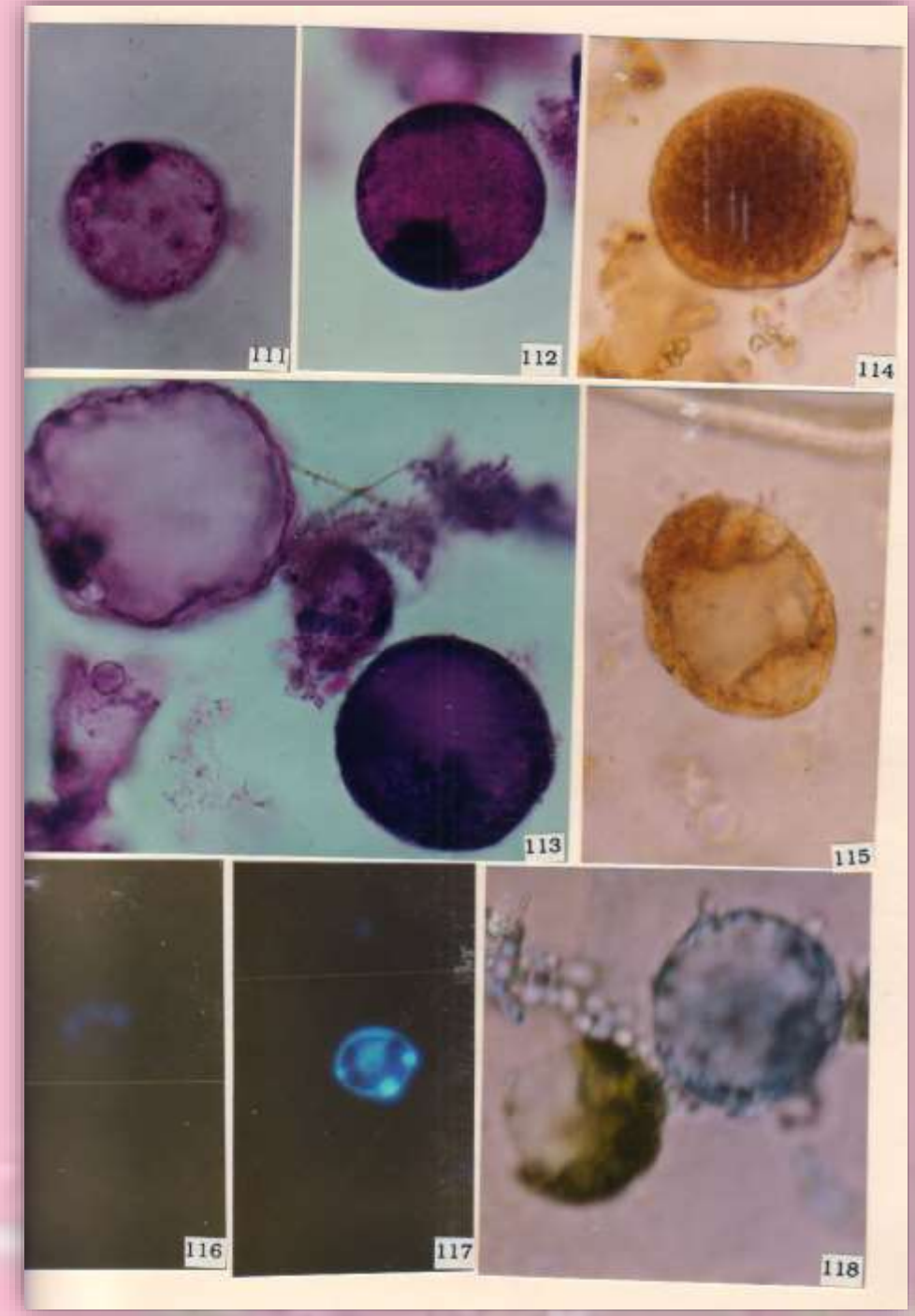
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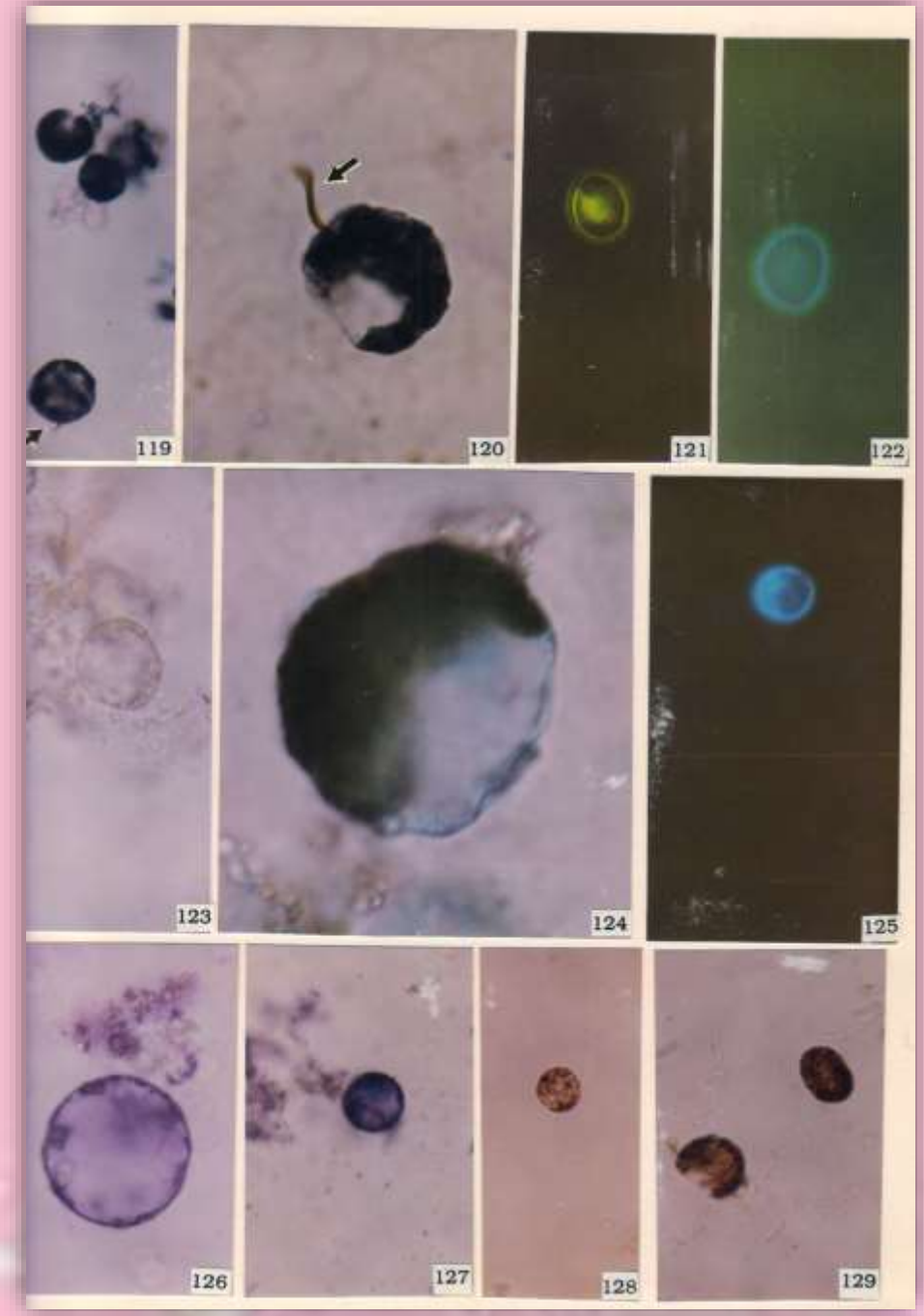
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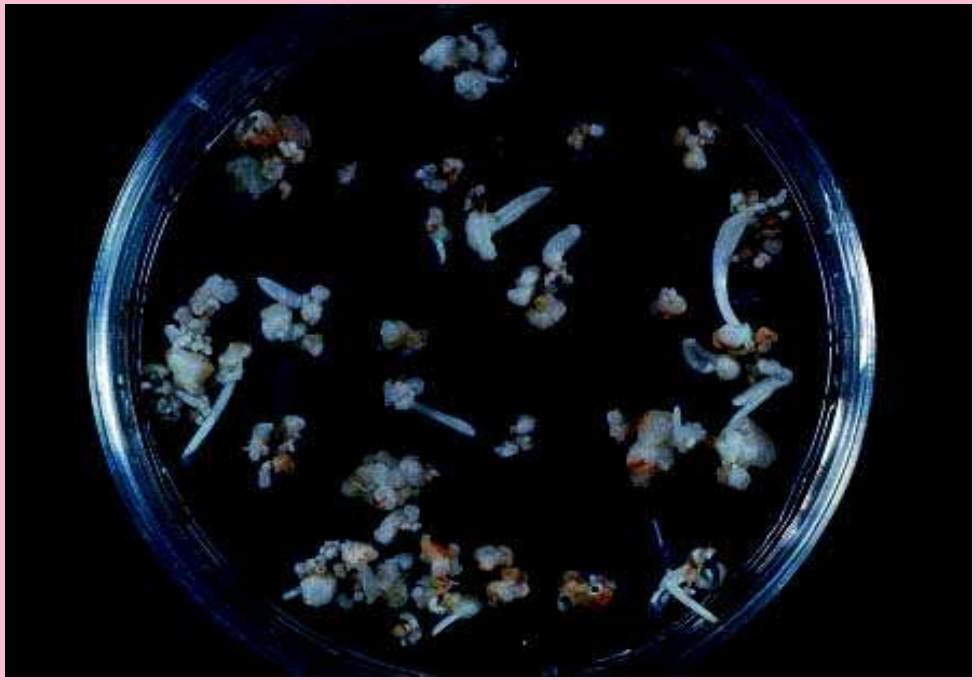
Banana plant regeneration from protoplast derived embryos



Protoplast Fusion

1. Mixing of protoplasts of two different genomes
2. Spontaneous fusion: from callus tissue, do not regenerate in to whole plants
3. Induced fusion by

- Polyethylene glycol method (PEG): high yield
- Nano3 (sodium nitrate)
- Ca2+ at high pH
- Electrofusion

4. Three main phases: agglutination or adhesion, plasma membrane fusion at localized sites, fused protoplasts

Method I of protoplast fusion (Polyethylene glycol (PEG) 6000)

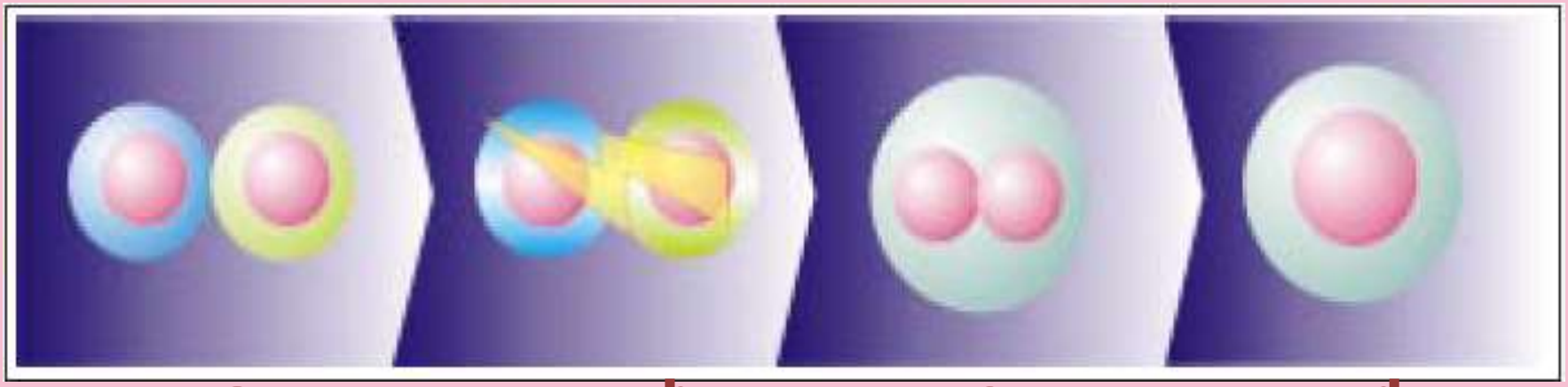
- The function of the PEG is to alter the membrane characteristics so that the protoplasts become sticky and if the protoplasts are allowed to come into contact they will adhere together and the contents will fuse
- Protoplasts isolated from each parent source to give a 4 ml suspension of protoplasts with a cell density of approximately 2 x 10^5 in CPW plus 13% mannitol are mixed then centrifuged at 100g for 10 min so as to leave the protoplasts in approximately 0.5 ml of medium
- The outer membranes are destabilized by adding 2 ml of PEG (30% PEG 6000, 4% sucrose, 0.01M $CaCl_2.6H_2O$ and autoclaved) and left for 10 min

- The protoplasts are fused by diluting the PEG every 5 min by adding a protoplast culture medium (MS medium, 2.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP, 3% sucrose and 9% mannitol) as increasing volumes (0.5, 1.0, 2.0, 3.0, 4.0 ml per tube)
- The protoplasts are resuspended after every dilution by gentle shaking and the mixture is centrifuged at 100g for 10 min then the protoplasts are washed in the culture medium without PEG, centrifuged and resuspended in the same medium
- The protoplast suspension is then layered onto agrose medium or mixed with an equal volume of agarose medium, as described earlier, to stimulate wall formation and cell division

Method II of protoplast fusion (electrical fusion)

- The suspension of protoplasts due for fusion is placed between two electrodes
- A weak ac current (400,000 Hz, 1.5V) is passed through the electrodes which causes the protoplasts to become positiviely charged on one side and negatively charged on the other
- As a result of their charge, the protoplasts align themselves in groups along the lines of force, touching one another and forming pearl chain

- The numbers in each chain can be altered by varying the protoplast density, the frequency of the ac field and the peak-to-peak voltage
- After a period of about 90s, the ac field is replaced by a single high voltage pulse discharge of for instance 1000 V cm^{-1}
- Where the protoplasts are in contact, the plasma membranes break and fuse forming a continuous membrane around the pearl chain
- This is then followed by cytoplasmic fusion



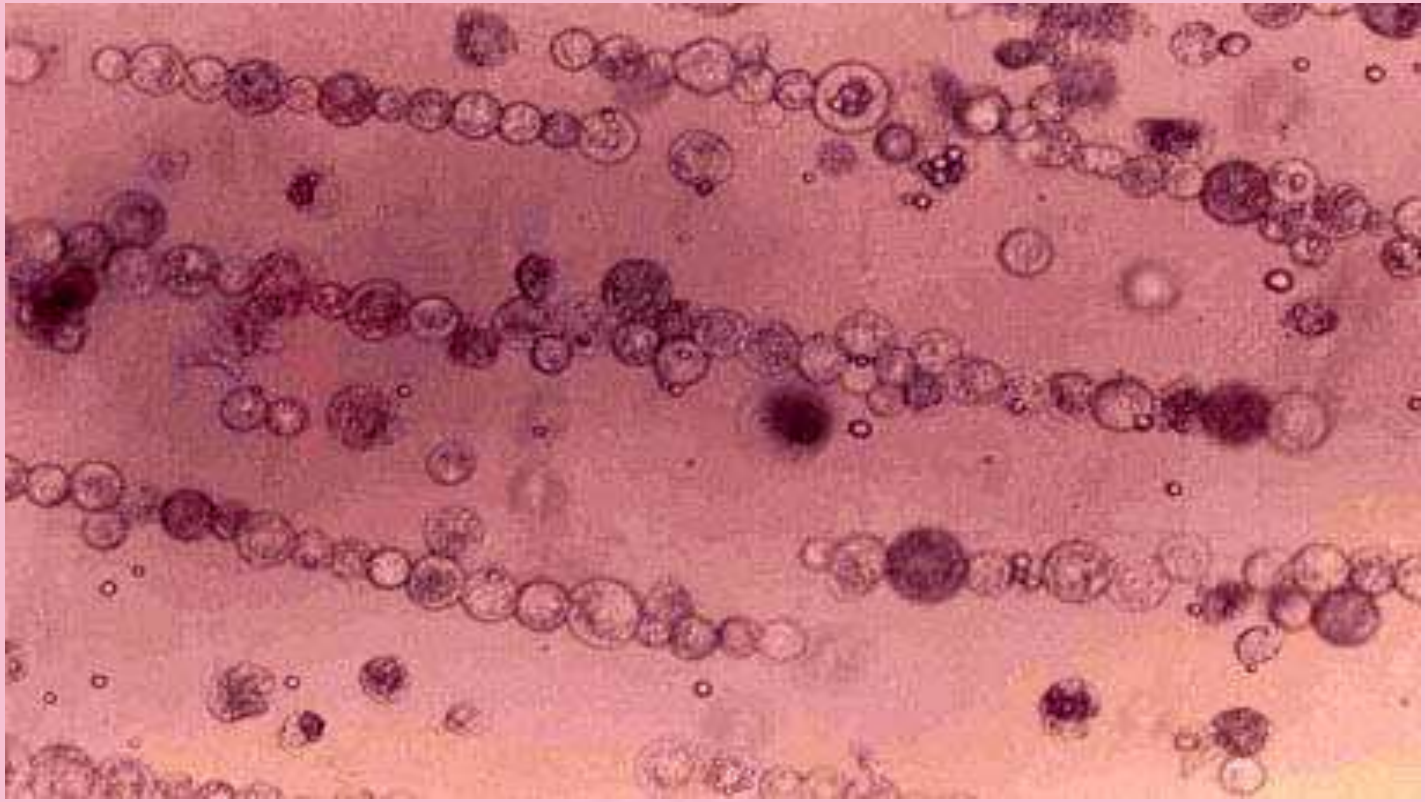
a) **Alignment:** Cells are brought into close contact by means of dielectrophoresis

b) **Fusion pulse:** A squarewave pulse of a mere 15 microseconds is applied in order to permeate the membrane. The membranes then fuse

c) **Heterokaryon phase:** The cell membranes are fused completely and the cytoplasm has mixed together. Only the nuclei remain separate.

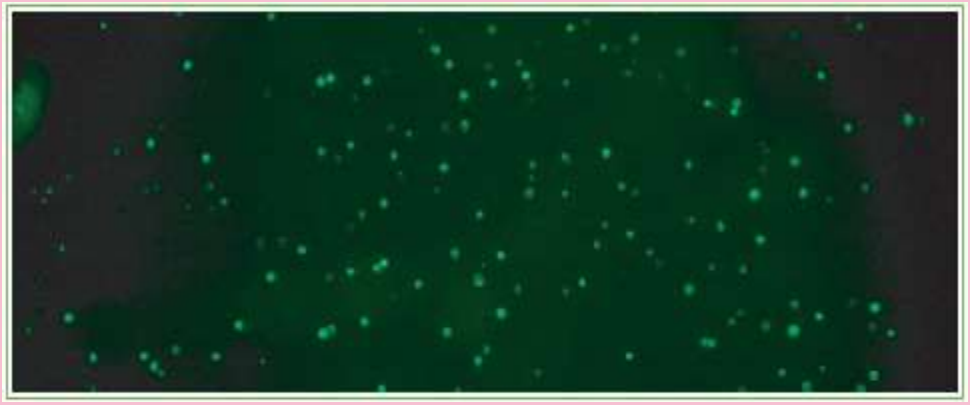
d) **Entire fusion product:** The nuclei are now fused as well. As a rule, the number of chromosomes is reduced.

PROTOPLAST CHAINS DURING ELECTROFUSION

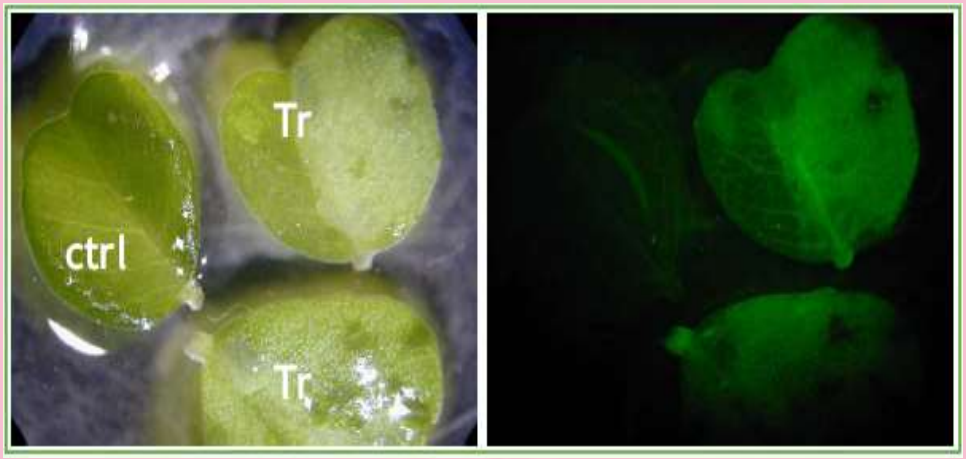


Selection of heterokaryons

- The simplest technique is to allow all of the protoplasts to regenerate and then identify the heterokaryon at the seedling or mature plant stage by morphological differences (efficiency is good)
- A straightforward but laborious technique is where the heterokaryons are identified visually then removed manually using a microscope and micromanipulator (efficiency is not good or the fusion of parent protoplasts with easily visual markers)
- The other popular but more expensive method uses an automated flow cytometer



Visualisation of transient expression in protoplasts transformed with GFP



Stable GFP expression in leaves in transgenic plants obtained after protoplasts transformation and regeneration of whole plant.

FACTORS INFLUENCING THE PROTOPLAST FUSION

Genotype:

- Frequency of regeneration and somaclones Explant source

Duration of cell culture:

- Most long-term cultures are chromosomally variable

Culture conditions:

- 2,4-D, NAA, BAP
- *In vitro* selection from suspension culture/protoplasts/calli

Examples of the applications of plant protoplasts

Plant name	Application
Arabidopsis thaliana	Gene recognition mechanisms involved in plant pathogenicity
A.thaliana / Zea mays	Elucidation of plant signal transduction mechanisms
Brassica chinensis	Electrophysiological studies of outward K+ channels
Bryopsis plumosa (marine green alga)	Electrochemical assays of metabolic flux; enzyme (peroxidase) activity
Cucurbita pepo	Viral pathogenicity
Helianthus annuus	Synthetic peptide import through the plasma membrane
Hibiscus cannabinus	Viral replication processes
Hordeum vulgare	Comparison of stress mechanisms in plants vs. human cancer cells

Nicotiana benthamiana	**Viral recombination and replication**
Nicotiana plumbaginifolia	**Genetic basis of developmental regulation and specificity**
Nicotiana tabacum	**Regulation of osmotic water transport across cell membranes**
Oryza sativa/Pisum sativum/ Sorghum bicolor/Triticum vulgare/ Z. mays	**Membrane permeability and tolerance to Al3+**
Phaseolus vulgaris	**Electrophysiological studies of inward-rectifying K+ channels**
Raphanus sativus	**Immunocytochemical evaluation of aquaporin accumulation**
Vicia faba	**Fluorometric analysis of photosynthetic electron transport**
Vigna radiata	**Intracellular responses to drought and salinity stress**
V. unguiculata	**Studies on plasma membrane organisation**
Z. mays	**Transient gene expression and proteomics**

Thank YOU

Eve...