

Direct gene transfer methods in plants (Microprojectile bombardment, Electroporation; polyethylene glycol (PEG)-mediated gene transformation, Silica carbon fibres whiskers), Transformation of protoplasts with naked DNA

Mitesh Shrestha

# Direct gene transfer in plants

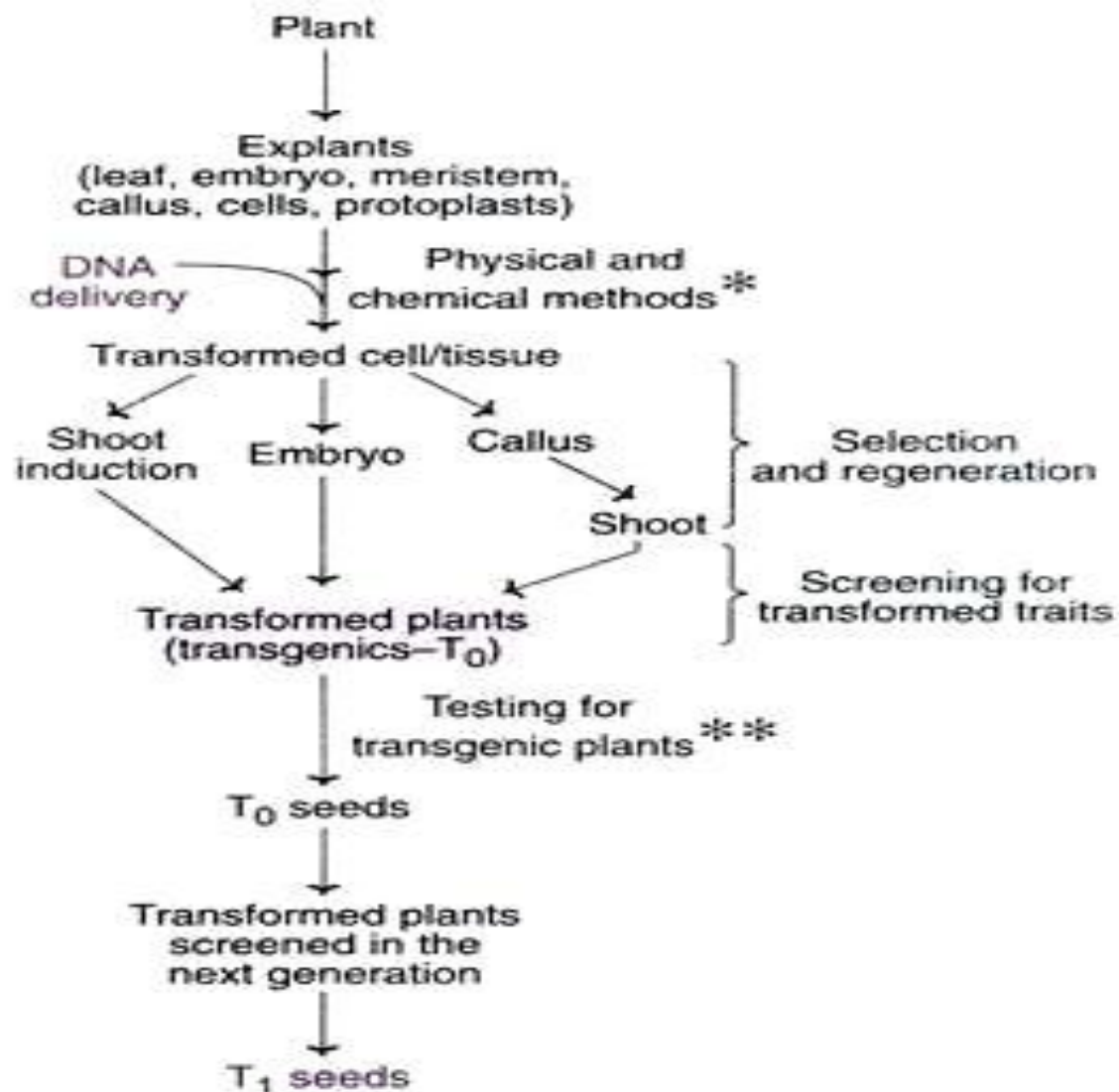
- Simple and effective
- Foreign DNA is directly introduced into the plant genome.
- Rely on the delivery of naked DNA into the plant cells.
- useful for both stable transformation and transient gene expression.
- However, the frequency of stable transformation is low, and it takes a long time to regenerate whole transgenic plants.

# Limitations of direct DNA transfer

- Frequency of transgene rearrangements is high.
- Results in
  - higher transgene copy number, and
  - high frequencies of gene silencing.
- Frequency of stable transformation is low, and it takes a long time to regenerate whole transgenic plants.

# Types of direct DNA transfer

- Physical gene transfer methods
  - Electroporation,
  - Particle bombardment,
  - Microinjection,
  - Liposome fusion,
  - Silicon carbide fibres.
- Chemical gene transfer methods
  - Polyethylene glycol (PEG)-mediated,
  - diethyl amino ethyl (DEAE) dextran-mediated,
  - calcium phosphate precipitation.
- DNA imbibition by cells/tissues/organs.



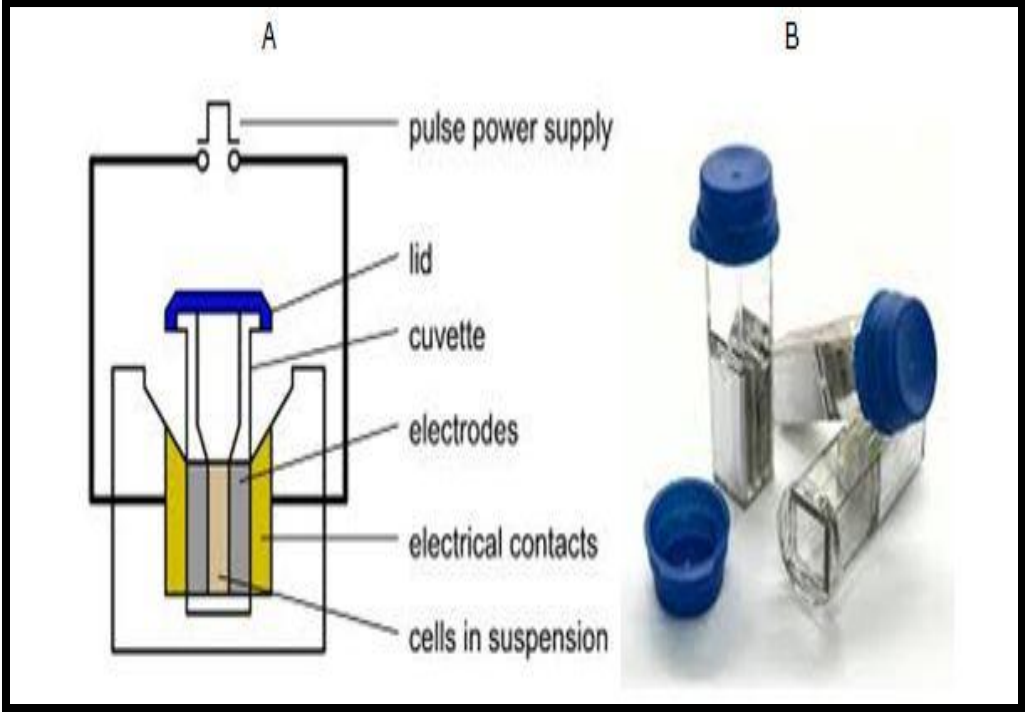
**Fig. 49.9** : An overview of the protocol for the production of transgenic plants using direct DNA delivery methods (\*Electroporation, microinjection, macroinjection, bombardment, etc.; \*\*Polymerase chain reaction, Southern hybridization)

# Electroporation

- Involves the use of **high field strength electrical impulses** to reversibly permeabilize the cell membranes for the uptake of DNA.
- Can be used for the delivery of DNA into **intact plant cells and protoplasts**.
- Plant material and foreign DNA are placed in a buffer between two electrodes and a high intensity electric current is passed, the alternating current of about 1 MHz is applied to align the protoplast by dielectrophoresis.

# Electroporation

- Once aligned, fusion is induced by applying one or more direct current pulses (1-3kV /cm), then the alternating field is reapplied briefly to maintain close membrane contact for fusion.
- Electric field damages membranes and results in the formation of pores in the plasma membrane through which DNA enters and gets integrated into the host cell genome.
- Technique is optimized by using appropriate electric field strength (defined as the applied voltage divides by the distance between two electrodes).
- Electroporation has been successfully used for the production of transgenic plants of many cereals e.g. **rice, wheat, maize**.

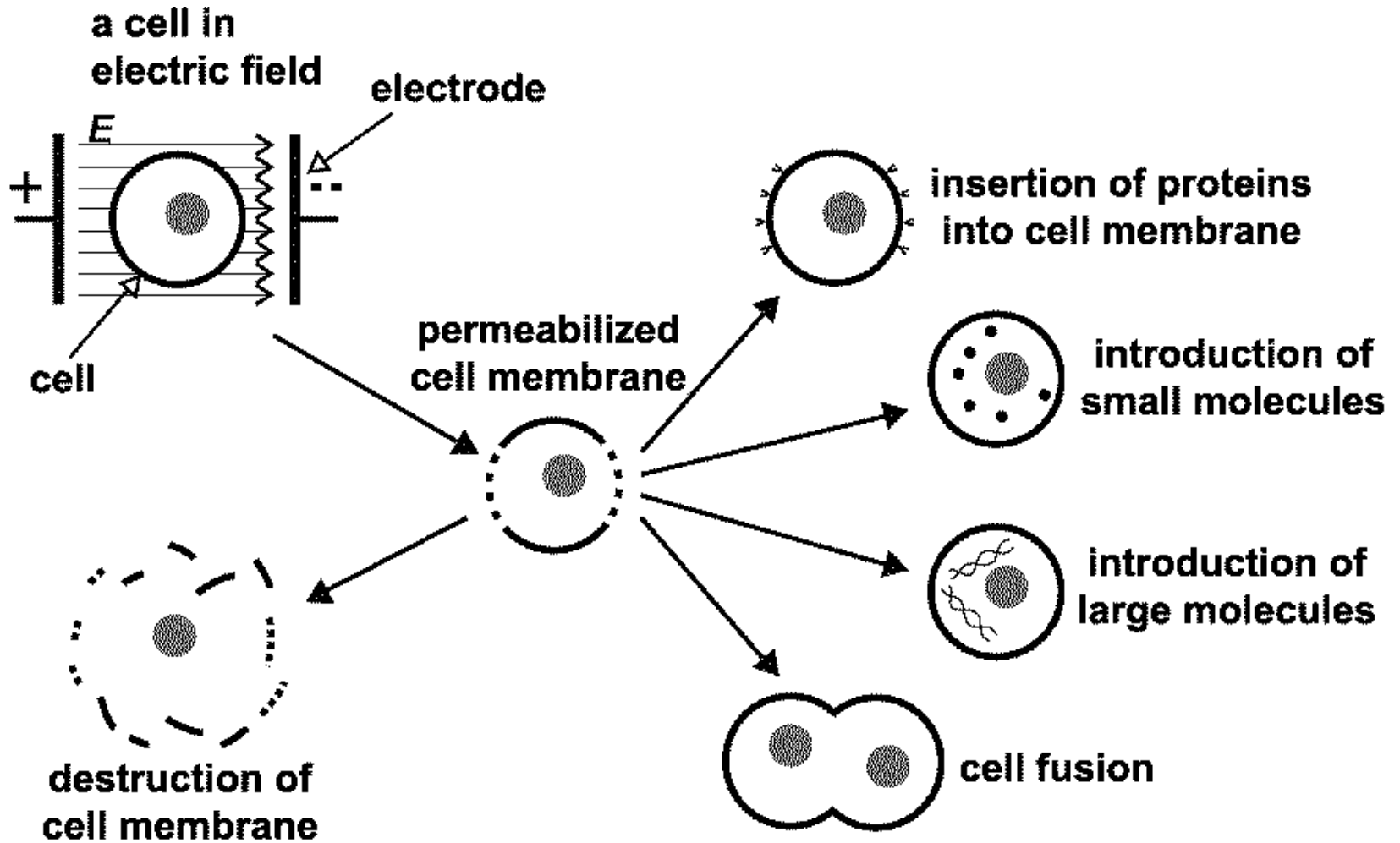




# Optimum field strength

- The pulse length of electric current
- Composition and temperature of the buffer solution
- Concentration of foreign DNA in the suspension
- Protoplasts density, and
- Size of the protoplasts.

# Electroporation



# Advantages of electroporation

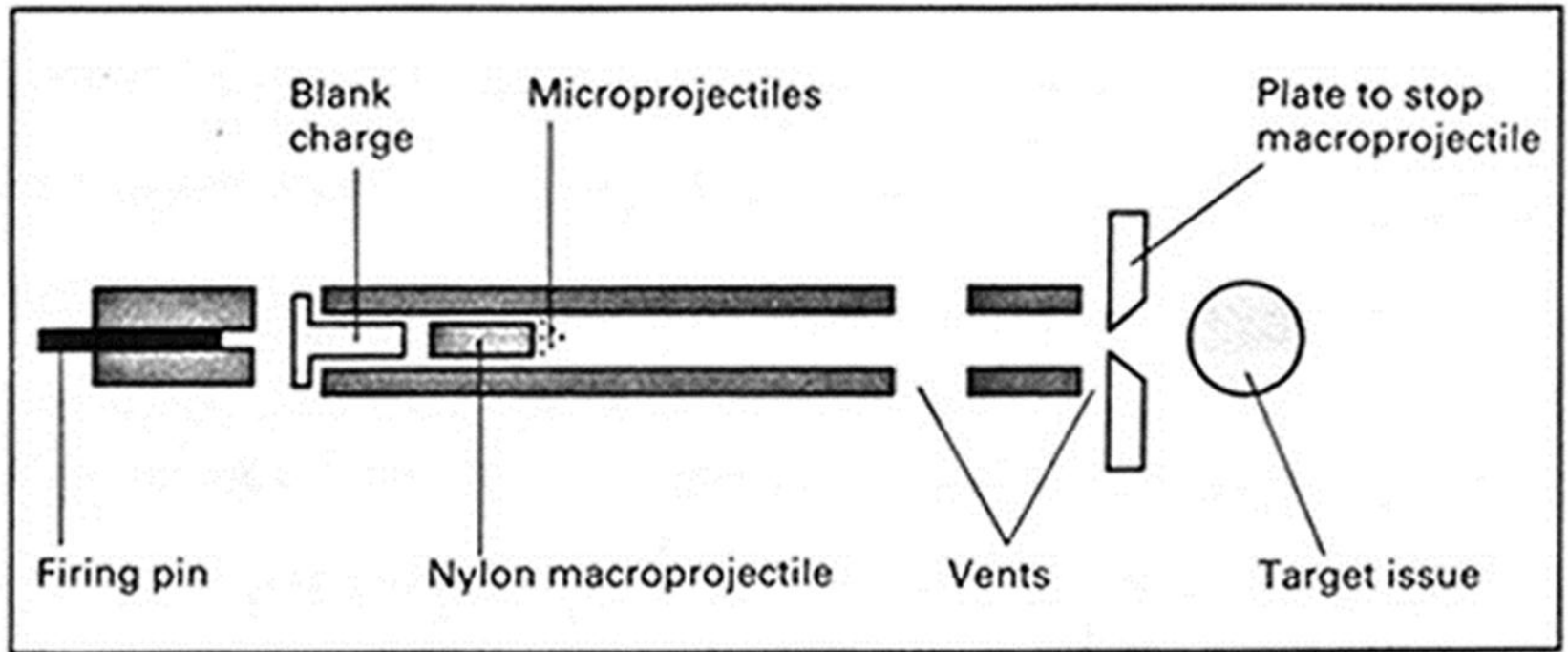
- **Simple, convenient and rapid, cost-effective.**
- The transformed cells are at the **same physiological state** after electroporation.
- **Efficiency of transformation can be improved** by optimising the electrical field strength, and addition of **spermidine**.
- ~40 to 50% incubated cells receive DNA
- ~50% of the transformed cells can survive

# Limitations of electroporation

- Under normal conditions, the amount of DNA delivered into plant cells is very low.
- Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.
- Regeneration of plants is not very easy, particularly when protoplasts are used.
- If the pulses are of wrong intensity, the pores may become very large or even fail to close causing the cell rupture
- Since the transport is non-specific-might result in ion imbalance that might later be toxic to cells (Weaver, 1995)

# Particle bombardment

- Also known as Biolistics, Particle gun, gene gun, bio blaster or micro projectile bombardment.



# Particle bombardment

- Introduction of DNA into the cells using microprojectiles
- Literally, DNA coated particles shot into target cells- payload is elemental particle of heavy metal coated with plasmid DNA
- Foreign DNA coated onto surface of minute gold or tungsten particles (1-3um)
- Used for stable transformation and transient expression
- Two types of cells commonly used for particle bombardment:  
Primary explants and the proliferating embryonic tissues

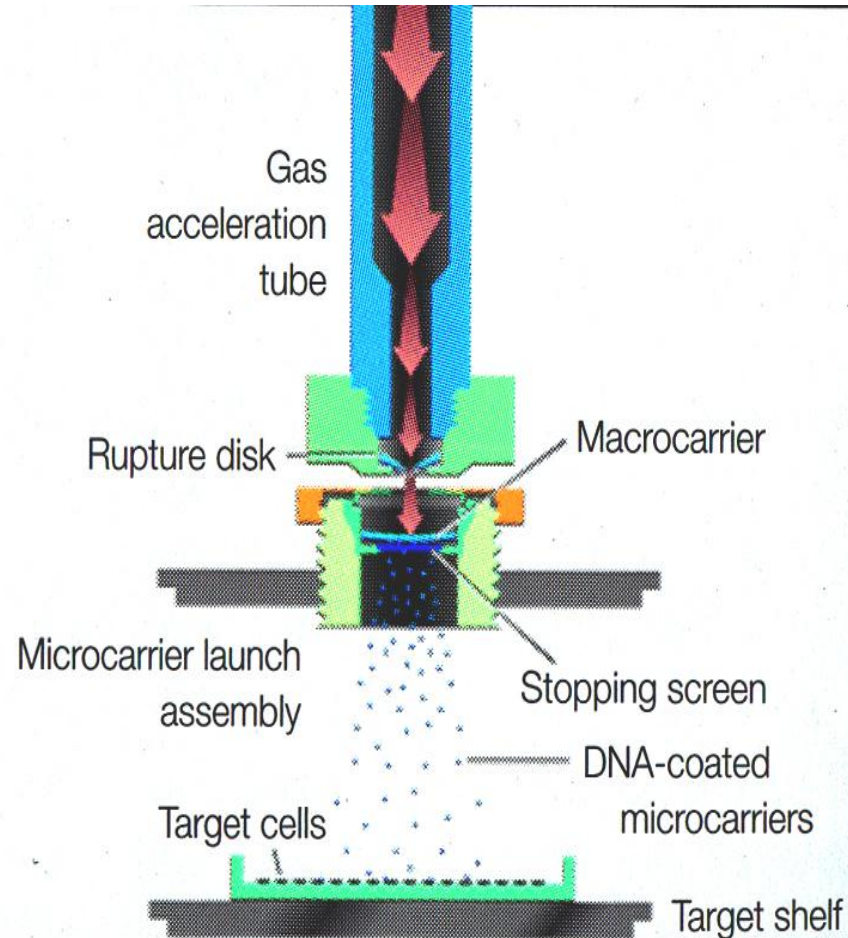


# Method:

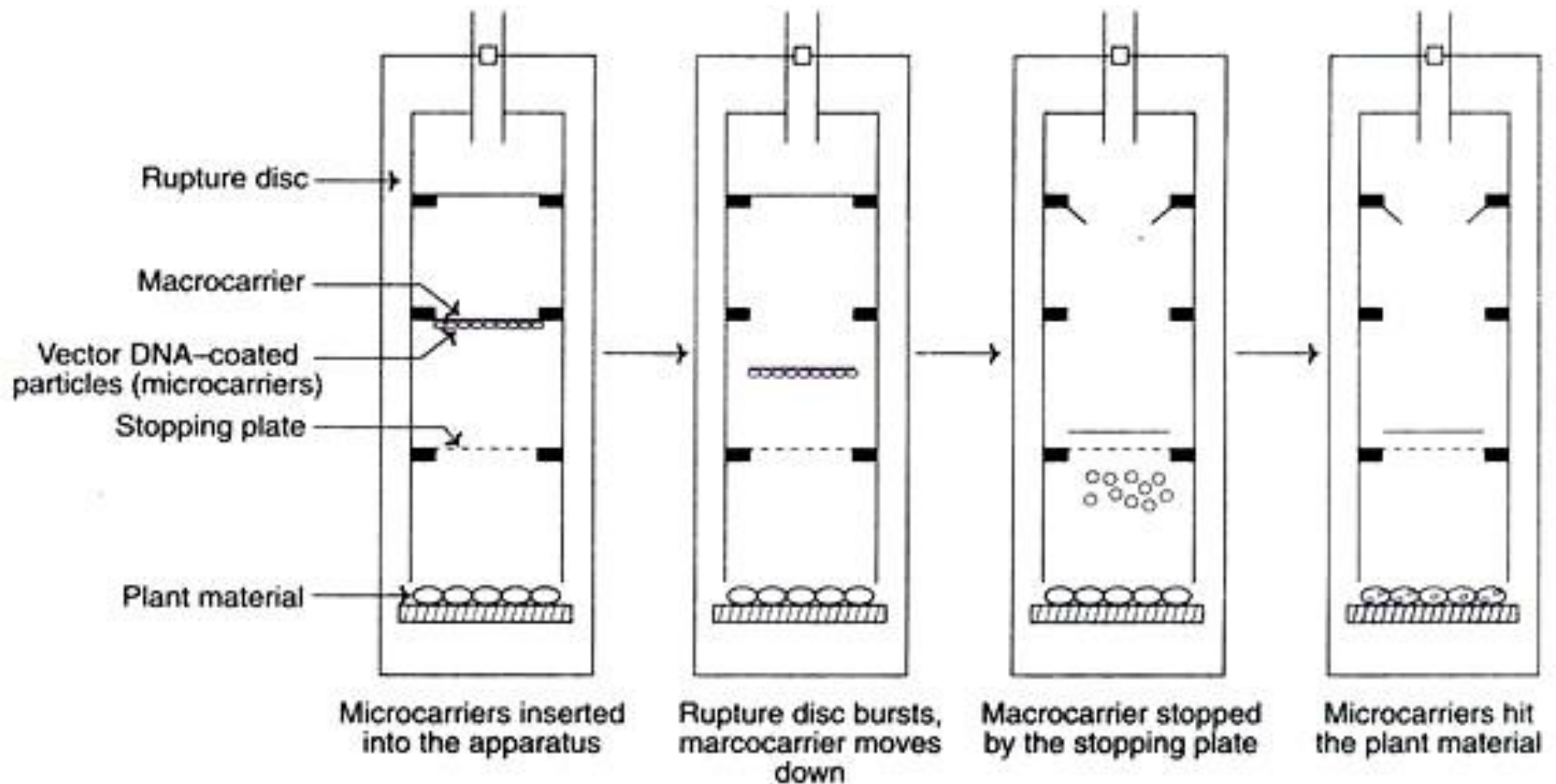
1. Precipitate DNA onto small tungsten or gold particles.
2. Accelerate particles to high speeds and aim them at cells or tissues.
3. Selective growth and regeneration of transgenic plants as described for Agro-mediated transformation

# Mechanism

- Microcarriers i.e. the tungsten or gold particles coated with DNA are carried by macrocarriers
- These macrocarriers inserted in to apparatus and pushed downward by rupturing the disc
- Stopping plate does not permit movement of macrocarriers while the microcarriers propelled at a high speed into the plant material
- DNA segments released which enter the plant cell and integrate into the genome







**Fig. 49.10 :** A diagrammatic representation of particle bombardment (biolistics) system for gene transfer in plants.

**TABLE 49.2 A selected list of transgenic plants (along with cell sources) developed by microprojectile bombardment**

<i>Plant</i>	<i>Cell source(s)</i>
Rice	Embryonic callus, immature zygotic embryos
Wheat	Immature zygotic embryos
Sorghum	Immature zygotic embryos
Corn	Embryonic cell suspension, immature zygotic embryos
Barley	Cell suspension, immature zygotic embryos
Banana	Embryonic cell suspension
Sweet potato	Callus cells
Cotton	Zygotic embryos
Grape	Embryonic callus
Peas	Zygotic embryos
Peanut	Embryonic callus
Tobacco	Pollen
Alfalfa	Embryonic callus

# Factors affecting bombardment

- Nature of micro particles
- Nature of tissues/cells
- Amount of DNA
- Environmental parameters

## **Advantages:**

- No need of specialized vectors
- High efficiency transient expression of foreign genes into the plants
- Theoretically unlimited host range ( applicable to all plants)

## **Disadvantages:**

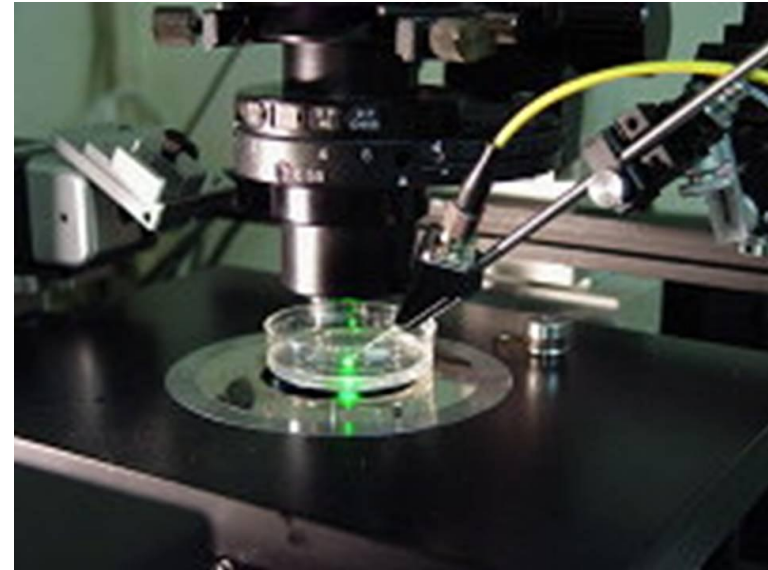
- Integrated DNA extensively rearranged (high copy number, extensive recombination- might lead some mitotic or meiotic instability)
- Target tissue may get damaged due to lack of control to bombardment velocity
- Sometimes, undesirable chimeric plants may be regenerated.

# Microinjection

- Direct physical method involving the **mechanical insertion** of the desirable DNA into a target cell.
- Labor intensive procedure that requires special capillary needles, pumps, micromanipulators, inverted microscope and other equipment.
- Used for the transfer of cellular organelles and for the manipulation of chromosomes.
- Selection of transformants by drug resistance or marker genes may be avoided
- In order to microinject protoplasts or other plant cells, **the cells need to be immobilized**
- Intact cells, protoplasts, callus, embryos, meristems etc.

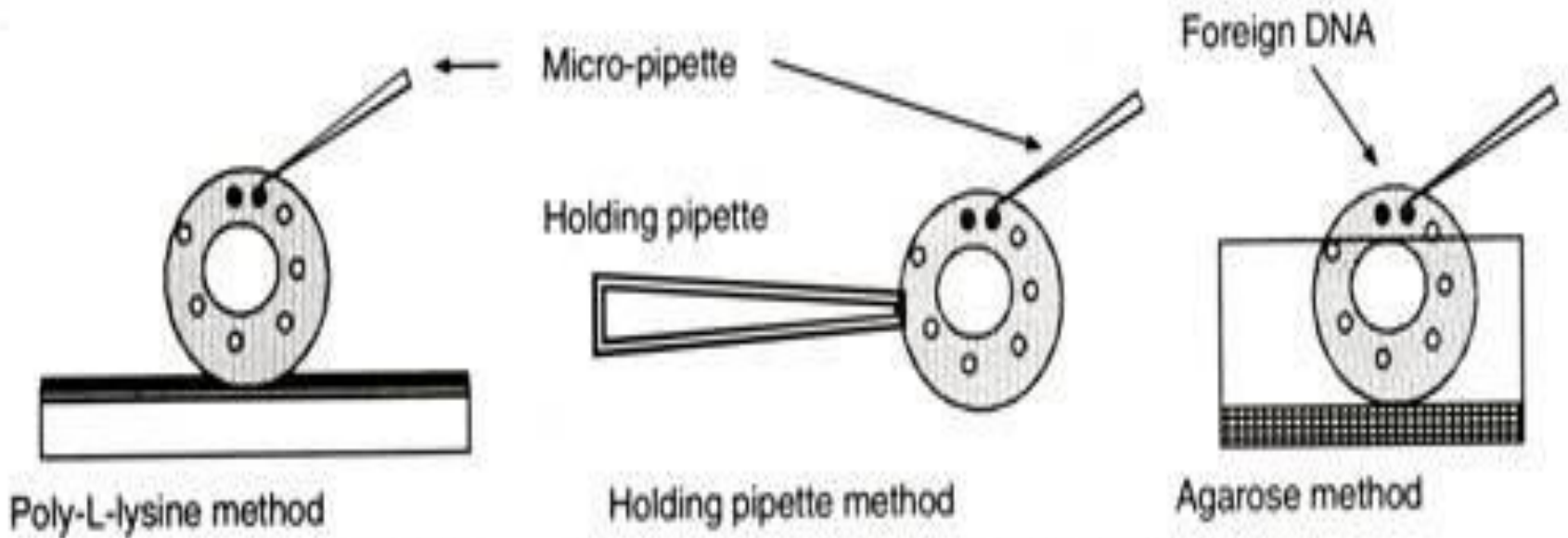
# Microinjection:

- Microinjection method uses fine needle to inject a solution of DNA directly into the target cells without damaging them
- Needles are usually 0.5 – 5.0  $\mu\text{m}$  in diameter
- Normally performed under a specialized optical microscope setup called **micromanipulator**
- Insertion of genetic material into a single cell to the specific site
- Protoplasts are immobilized on agarose embedding or agar embedding or on glass slides coated with poly-lysine or by holding them under suction by a micropipette
- Once the injection has been completed the cells must be cultured carefully to ensure continued growth and development



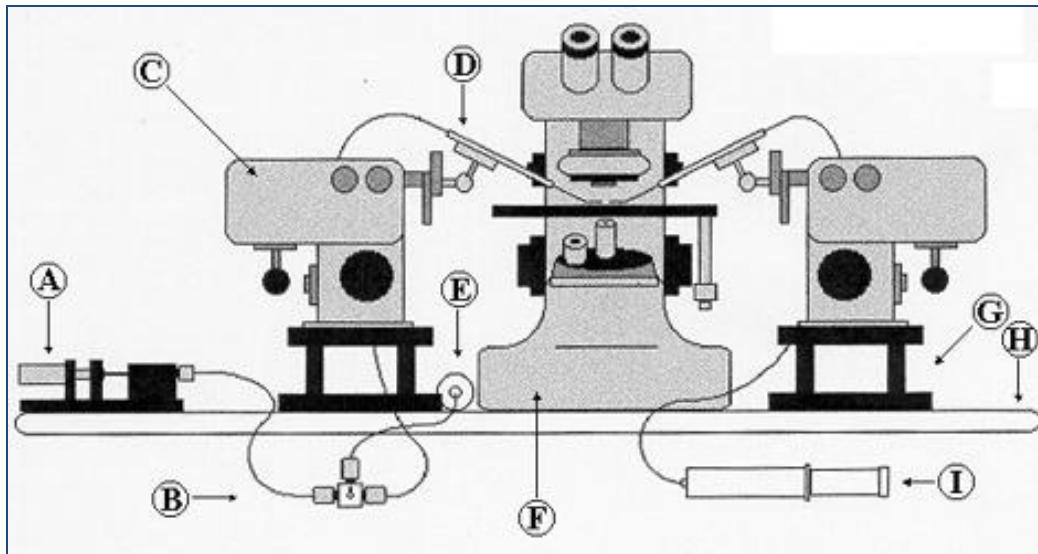
**Stereoscopic microinjection set-up**

# Cell Immobilization



**Fig. 16.3.** Various methods of immobilizing the cell and microinjection.

# Microinjection

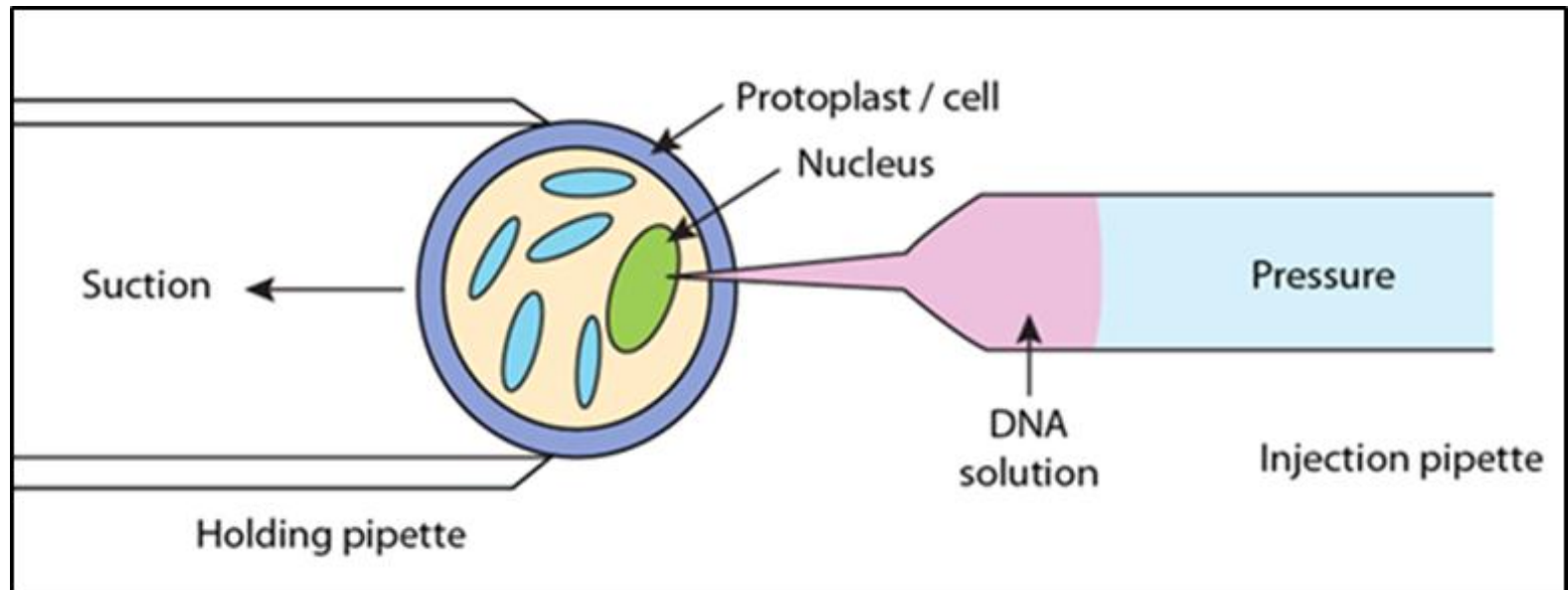


Microinjection

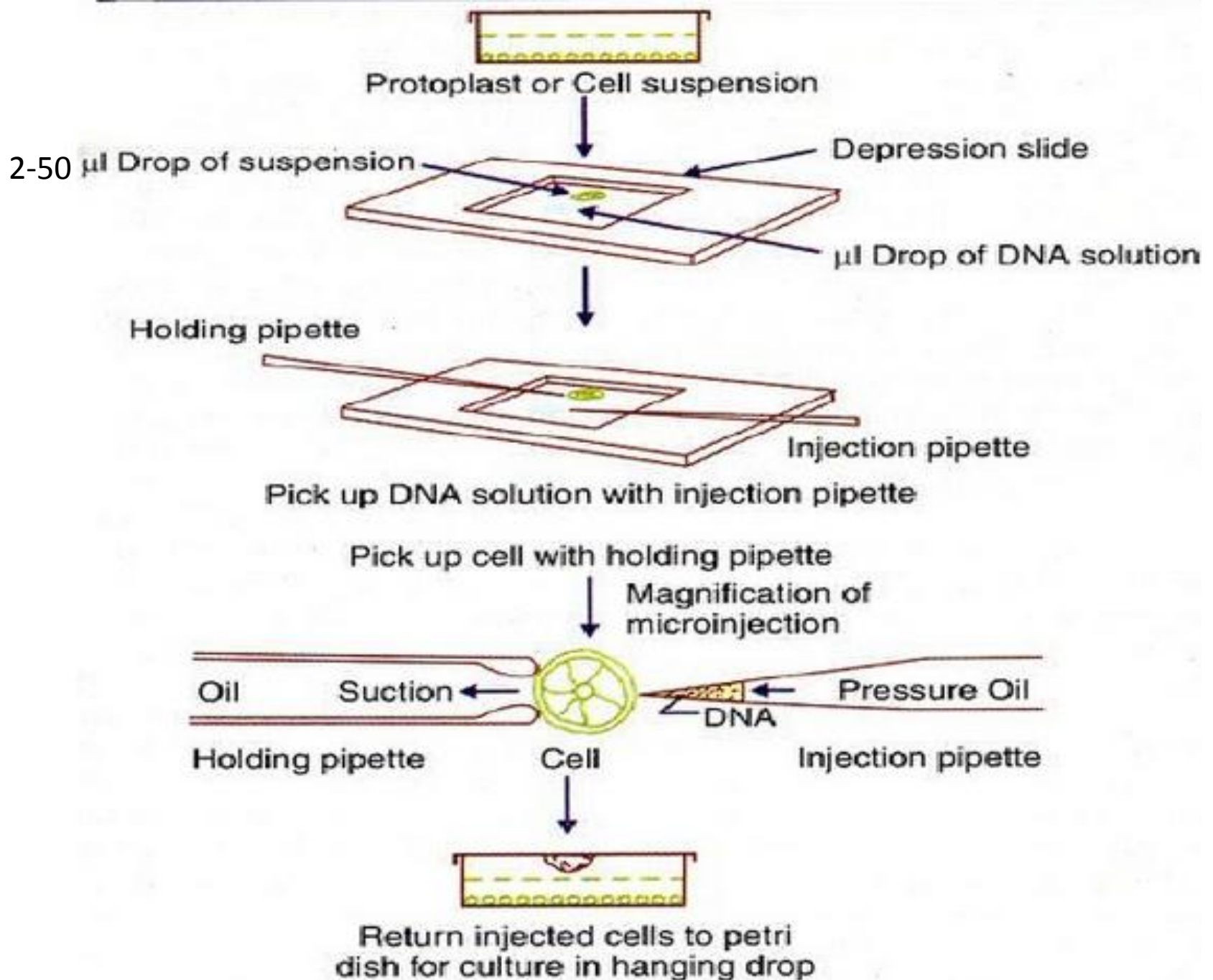




# Microinjection



- In order to manipulate the protoplasts without damage, the protoplasts are cultured for from about 1 to 5 days before the injection is performed to allow for partial regeneration of the cell wall.
- It was found that injection through the partially regenerated cell wall could still be accomplished and particular compartments of the cell could be targeted.
- Most plant cells are injected while keeping inside micro-droplets (2-50  $\mu$ l) of medium using a chamber which is sterile, vibration free and permits temperature and humidity regulation.
- A maximum of 100-200 cells per hour can be microinjected by this method.
- Successful transformation by microinjection of cells have been achieved in tobacco, alfalfa
- Well established for animal cells like in IVF techniques



**Fig: A general overview of Microinjection to protoplast cells**

## **Advantages:**

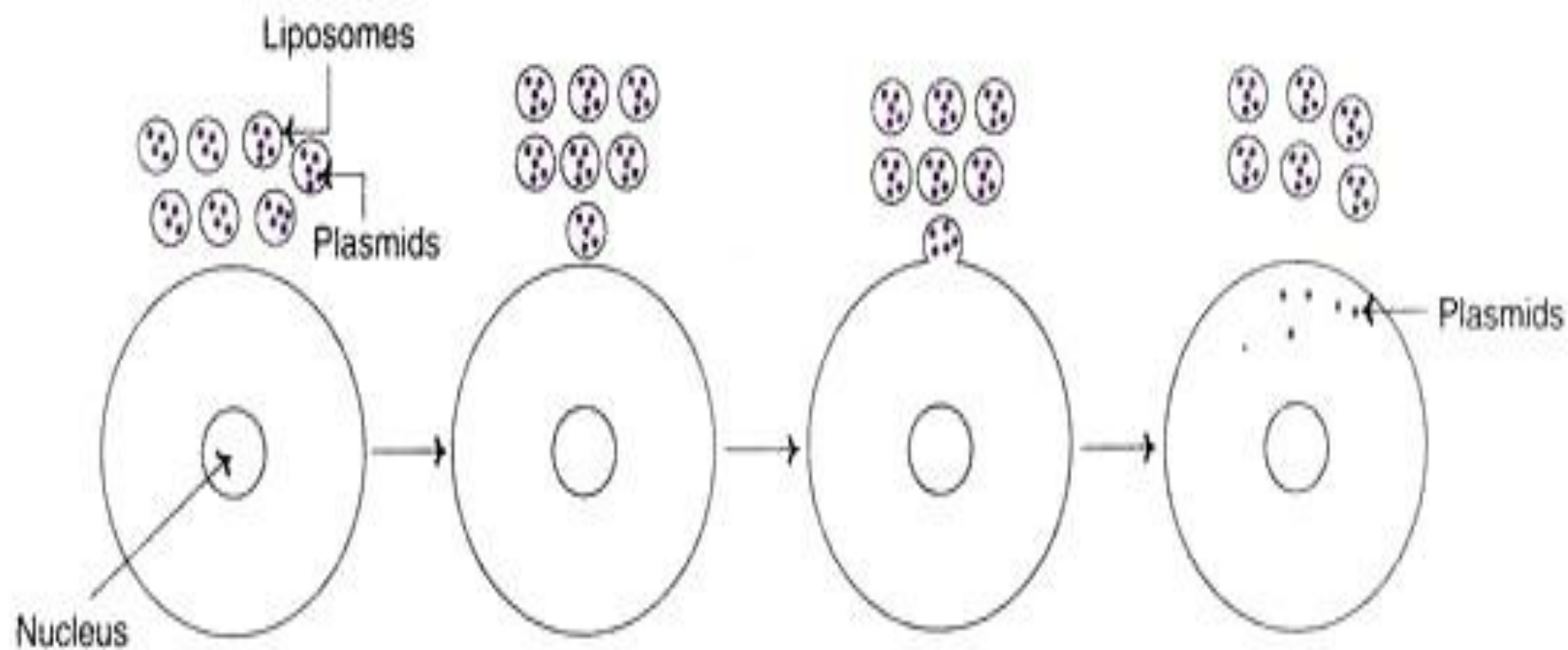
- Host range independent
- Does not necessarily require protoplast regeneration system as the techniques even allow successful transfer of gene or DNA directly into the defined cells within multicellular structure such as embryo, ovule and even meristem as well
- Chimeric plants (with only one part of the plant transformed) can also be generated
- High transformation efficiency as the operator can directly inject the DNA into the target nucleus

## **Disadvantages:**

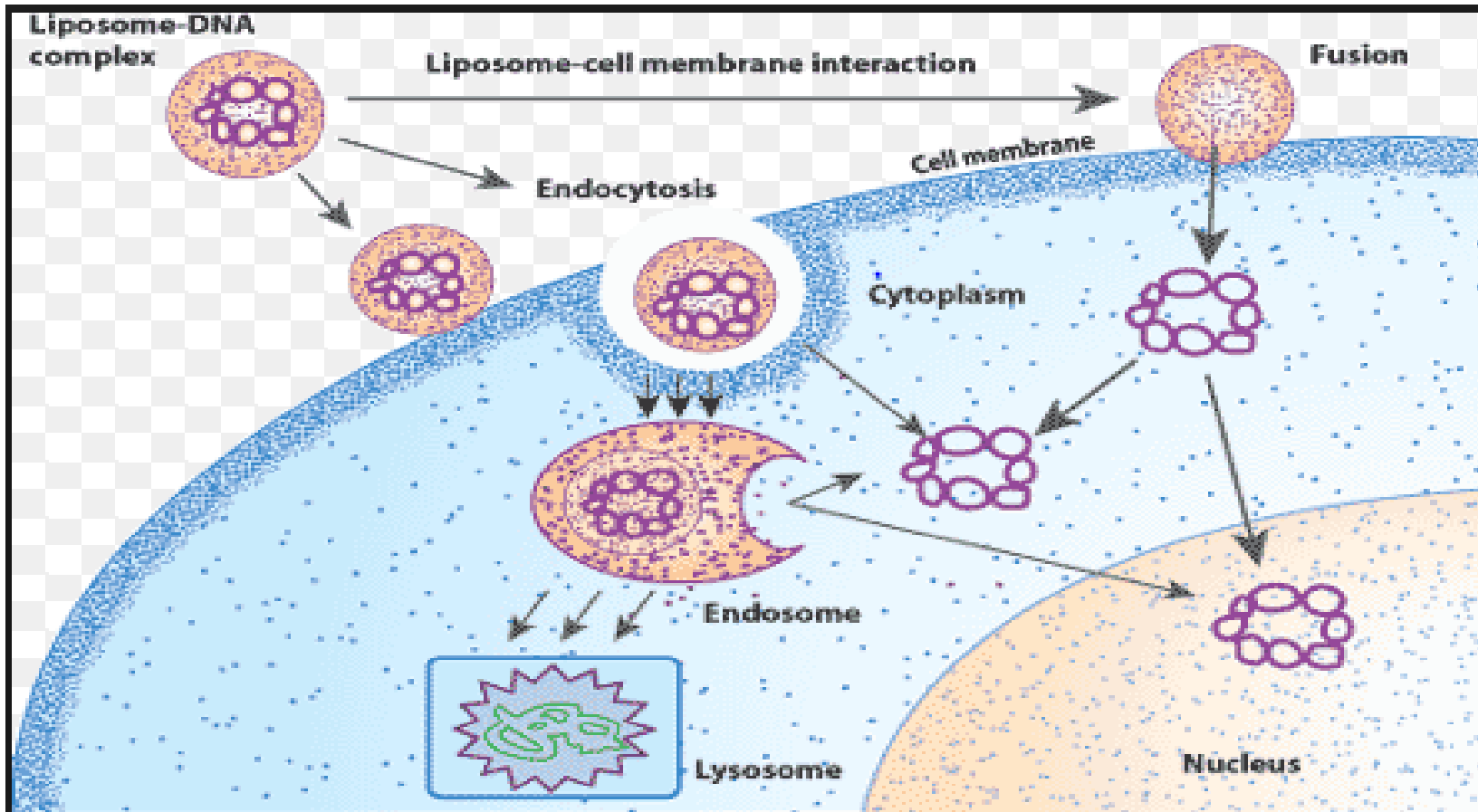
- Slow and expensive
- Requires highly skilled and experienced manpower

# Liposome-Mediated Transformation

- Liposomes are **artificially created lipid vesicles** containing a phospholipid membrane.
- They are successfully used in mammalian cells for the delivery of proteins, drugs etc.
- Liposomes carrying genes can be employed to fuse with protoplasts and transfer the genes.
- 3 steps
  - Involves adhesion of liposomes to the protoplast surface,
  - Fusion at the site of attachment and
  - Release of plasmids inside the cell



**Fig. 49.12 :** A diagrammatic representation of fusion of plasmid-filled liposomes with protoplasts.



# Advantages of liposome fusion

- Being present in an encapsulated form of liposomes, DNA is protected from environmental insults and damage.
- DNA is stable and can be stored for some time in liposomes prior to transfer.
- Applicable to a wide range of plant cells.
- There is good reproducibility in the technique.



# Limitations of liposome fusion

- Difficulty associated with the regeneration of plants from transformed protoplasts.

# Silicon carbide fibers: Whiskers

- Silicon carbide fibers- whiskers are very small hollow fibers of about **0.3-0.6  $\mu\text{m}$**  in diameter and **10-100  $\mu\text{m}$**  in length;
- Introduced into a buffer containing the DNA of interest and the target plant materials which and vortexed
- Obtained by thermal reduction of silica in reduced atmosphere
- Great intrinsic hardness with sharp cutting edges
- Proposed mechanism of DNA delivery: **function as numerous needles facilitating delivery of DNA by cell perforation and abrasion**
- Transformation of **rice, wheat, maize and barley**

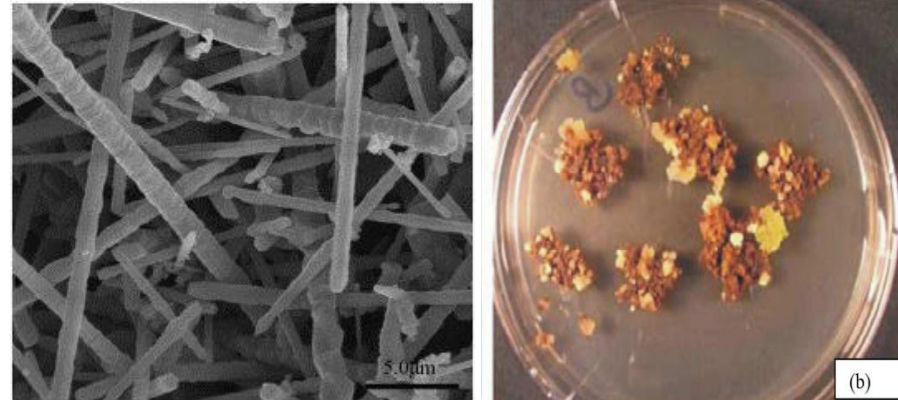


Fig: a) Silicon carbide whiskers  
b) induction of kanamycin resistant cotton calli from embryogenic calli transformed with SiC

## Advantages:

- Unlike particle bombardment and PEG treatment of protoplast, whiskers deliver plasmid DNA in non-precipitated form, making it possible for controlling the quantity of DNA available for transformation
- Able to transform walled cells thus avoiding the necessity of protoplast isolation- though cells in suspension culture, embryos and embryo derived callus are more preferred
- Also used in generation of wound thereby facilitating *Agrobacterium* mediated genetic transformation
- Rapid and inexpensive-low equipment cost as it doesn't require any special equipment

## Disadvantages

- The drawbacks of this technique relate to the availability of suitable plant material and the inherent dangers of the fibers, which require careful handling.
- Low transformation efficiency owing to tissue damage
- Many cereals, produce embryonic callus that is hard and compact and not easily transformed with this technique.
- Causes respiratory health hazard and even are found to be carcinogenic

# **Polyethylene glycol (PEG) mediated transformation method**

- Plant protoplast can be transformed with naked DNA by treatment with PEG in the presence of divalent cations e.g., Calcium.
- PEG and divalent cations destabilize the plasma membrane of the plant protoplast and rendered it permeable to naked DNA.
- DNA enters the nucleus and integrates into the host genome.

# Polyethylene glycol (PEG)

- Isolation of protoplasts
- Suspension of the isolated protoplast along with the plasmid DNA in a tube
- Addition of 40% PEG 4000 dissolved in mannitol and calcium nitrate
- Incubation for about 5 min
- Observation of gene transformation following plant regeneration

# PEG- mediated transformation of Protoplasts

## (Methods in Molecular Biology, vol.82: Arabidopsis protocols)

- Isolation of protoplasts from leaf mesophyll tissue, root and cell suspension cultures
- Wash the protoplasts with **0.45M mannitol** and resuspend the protoplast pellet in **1ml of mannitol-magnesium solution**
- Count the number of Protoplasts to adjust the density at  **$1 \times 10^6$  cells/ml**
- Place the **suspension on ice for 35min** and the **centrifuge at 60 g for 5min**
- Resuspend the pellet in **0.3ml of mannitol-Mg solution** and carefully transfer as single droplet in the middle of a glass petri-dish
- Slowly add **25-35  $\mu$ g of Plasmid DNA** dissolved in water into the protoplast drop and shake the petri-dish gently to ensure proper mixing
- After 5min add **0.3ml of PEG solution** at the circumference of the drop of the protoplast suspension
- Carefully mix the suspension by tilting the petri-dish gently
- **After 10min**, from the sides of the protoplast droplet, add **1ml of 0.45M mannitol**
- **At 2 min** interval add again **2ml of 0.45M mannitol** and gently shaken- this step repeated until the total volume is **about 12ml**
- Collect the resulting protoplast by centrifugation at **60g for 5min**
- Assay for transient gene expression or start embedding the protoplast into alginate and proceed further for plant regeneration

## **Advantages:**

- Simple, efficient, allowing simultaneous processing of many samples
- Yield transformed cell population with high survival and division rates
- Method utilizes inexpensive supplies and equipment
- Helps to overcome a hurdle of host range limitations of *Agrobacterium* mediated transformation

## **Disadvantages:**

- Plant protoplasts are not easy to work with and the regeneration of fertile plants from protoplasts is problematic for some species
- DNA used is also susceptible to degradation and rearrangement
- Integration at random sites



# Sonication

- Mild sonication (**20 KHz ultrasound**) has been used to facilitate the uptake and transient expression of a chloramphenicol acetyltransferase (CAT) gene in protoplasts of sugar beet (*Beta vulgaris*) and tobacco.
- This method was found superior than electroporation method used for the same material.
- Plating efficiency was also similar to untreated cells.
- However, transgenic plant production using this technique has not been reported so far.

# Transformation of protoplasts with naked DNA

- Electroporation
- Chemically stimulated DNA uptake by protoplasts
- Liposomes
- Micro-injection
- Sonication

# Assignment

- Explain about various modes of direct gene transfer methods in plants along with their advantages and disadvantages. [7.5]
- What are the advantages of direct mode of gene transfer to indirect one? [3]
- Write down the procedure for PEG mediated gene transfer in plants. [3]