Chhattisgarh Samvad

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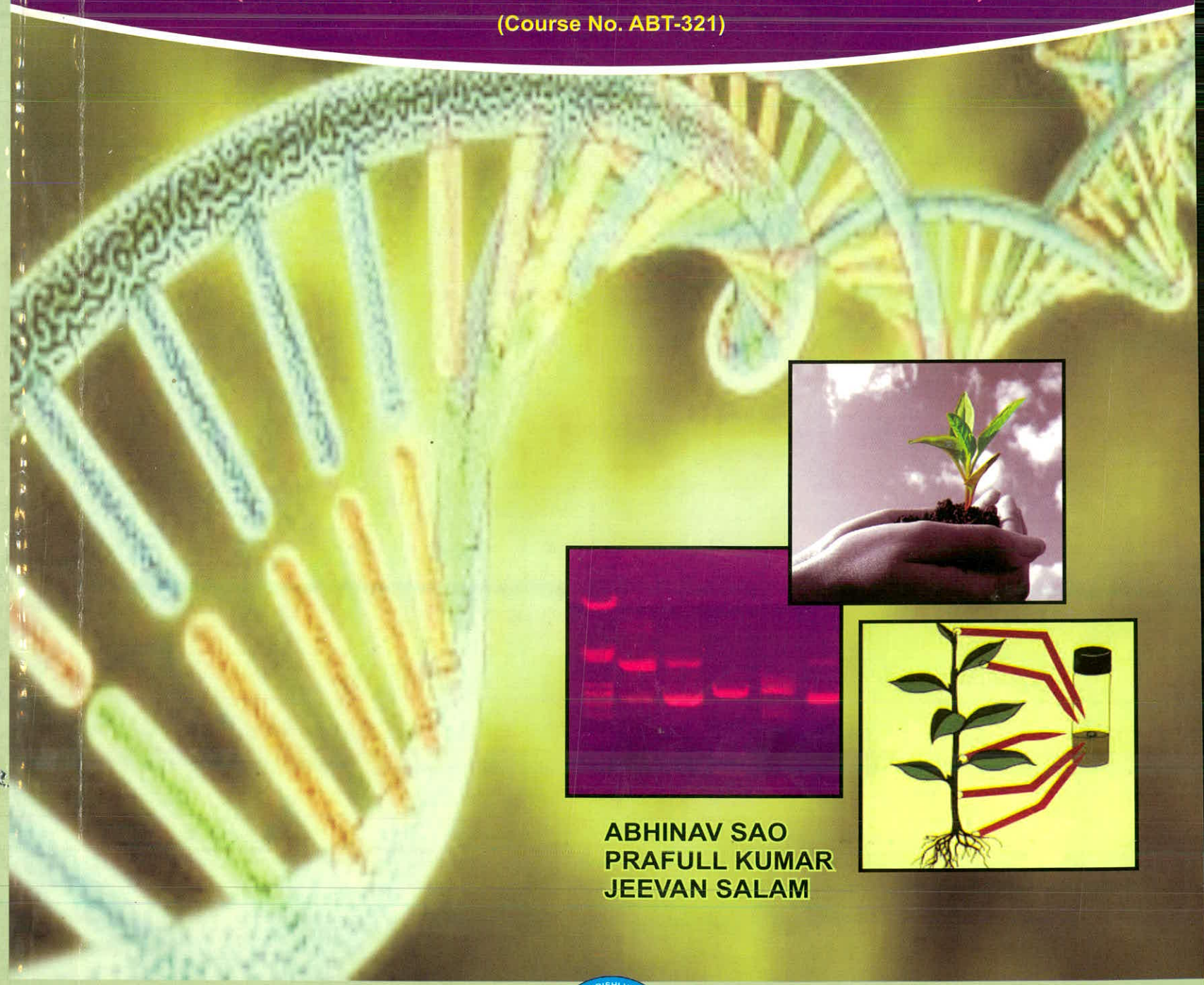
PRACTICAL MANUAL

PRINCIPLES OF BIOTECHNOLOGY

(FOR UNDERGRADUATE AGRICULTURE STUDENTS)

(Course No. ABT-321)



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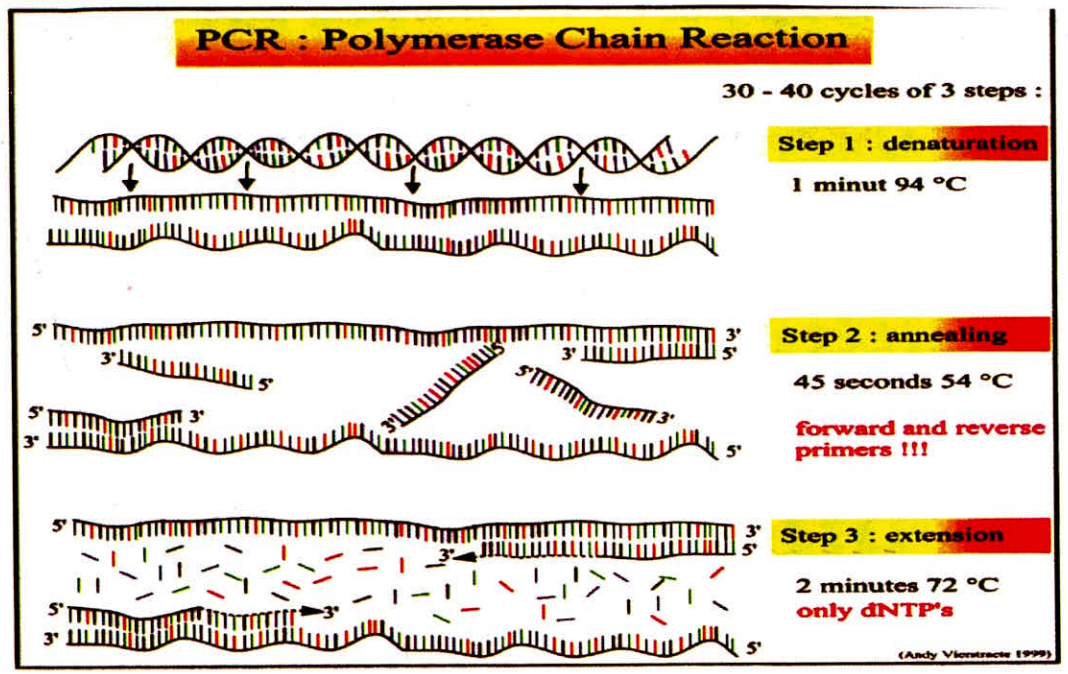
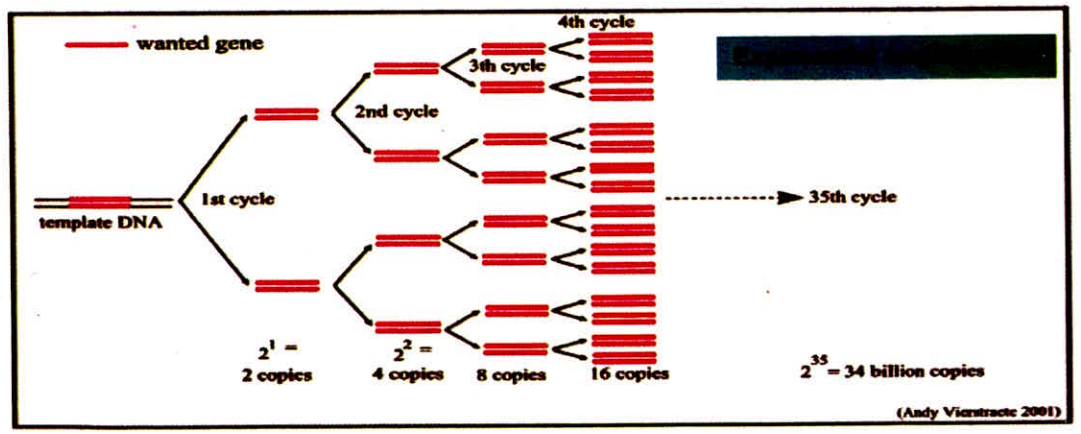


Estimation of Copy no. :

1 copy of gene before amplification

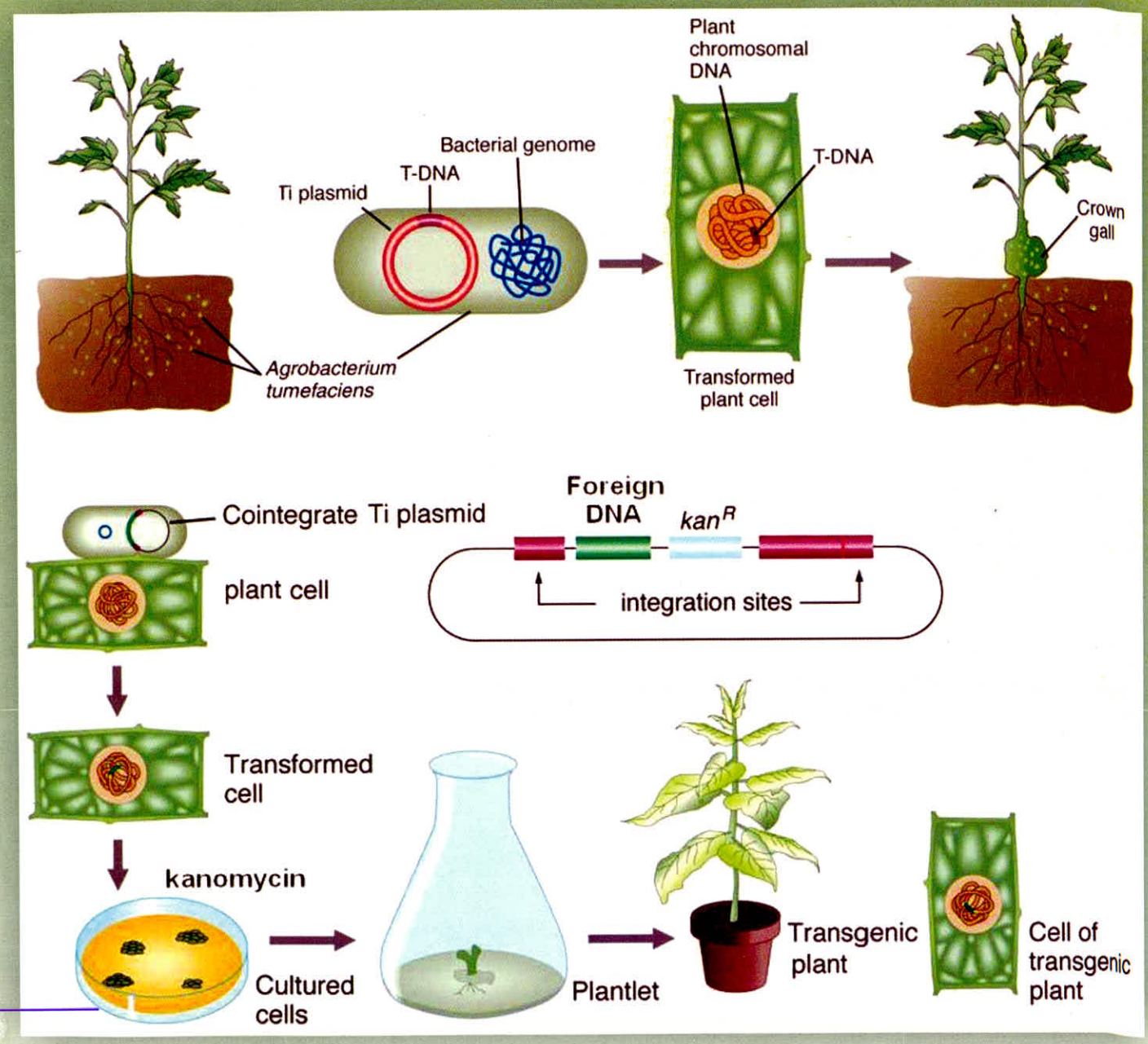
After 35 cycles it will be 2^{35}= 34 billion copies



PCR product yield = (input target amount) x (1 + % efficiency) X cycle number

% efficiency = amplification efficiency

Source: Karry Mullis, Presentation



Process of Transgenic Plant Development

इंदिरा गांधी कृषि विश्वविद्यालय

कृषक नगर, रायपुर 492 012 (छत्तीसगढ़) भारत

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Dr. S.K. Patil
Vice Chancellor

Message

It is matter of great pleasure that the S.G. College of Agriculture & Research Station, Kumhrawand, Jagdalpur is publishing Practical manual of Principles of Biotechnology for under Graduate Agricultures Students. This manual will provide information about practical aspects of recent tool of Genetics and Crop improvement i.e. tissue culture and molecular biology tool.

I wish every success for publishing "Practical Manual of Principles of Biotechnology" and also appreciate sincere efforts of Dean, staff member and contributors for bringing out such important publication for students.

(S.K. Patil)

इंदिरा गांधी कृषि विश्वविद्यालय

शहीद गुण्डाधुर कृषि महाविद्यालय एवं अनुसंधान केन्द्र

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Dean

Foreword

S.G. College of Agriculture & Research Station playing pivotal role in Agriculture Research & Education in Bastar plateau zone of chhattisgarh. Practical is the integral part of teaching of Agriculture students and Biotechnology is basically practical related course.

The teachers of Principal of Biotechnology course are bringing out Pratical Manual for this important course in recent era of advancement in the field of Agriculture Sciences. This will guide under graduate agriculture students in Biotechnological aspects of plants.

I would like to congratulate the authors of this manual for their efforts and sincerity to bring out this important publication for the benefit of students.

(S.C. Mukherjee)

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EXPERIMENT – 1

AIM : Requirements for Plant tissue Culture Laboratory.

The setting up of a complete/ functional Plant Tissue Culture Laboratory needs proper planning. A Tissue culture laboratory will be divided in to five distinct areas :

1. Media preparation room
2. Aseptic transfer chamber
3. Environmentally controlled culture room
4. Analytical room
5. Acclimatization room

Media preparation room : Laboratory tables and stools, hot plate, magnetic stirrer, analytical top pan balance (0.1 mg – 180 g $\pm$ 0.001 g and 100 mg – 500 g $\pm$ 0.1 g), Refrigerator and freezer, Water purification and storage system, Glassware washing facility, Hot Air oven, Microwave oven, pH meter, Autoclave. Cabinet or shelves for storage of glasswares, chemicals etc. and chemicals for culture media.

Aseptic Transfer chamber : Laminar air-flow, Micro-dissecting scissors, scalpel handles with blades, forceps (various sizes), needles and inoculating loops, gas outlet, vaccum facility, tissue paper/filter paper, sterilize and pipette dispenser.

Environmentally Controlled Culture Room : Racks with light arrangements on timers and controlled temperature (25 + 2^{0}C) maintained with Air conditionars, Incubators with dark and dark/light photoperiod controlled temperature, Rotary shaker of variable speed from 80-220 rpm to take 100 ml or 250 ml Erlenmeyr flasks with arrangement of lighting to provide an intensity of 2000 to 4000 lux. Lux meter to measure intensity of light.

Analytical Room : Inverted microscope, Colorimeter for chemical estimation such as chlorophyll, starch, nucleic acid, phenols, oxidizing enzymes etc., centrifuge, chemical reagent racks, viscocity meter, Gas outlet.

Acclimatization Room : The hardening chamber needs high illumination (4000-10,000 lux) and high humidity (90-100 % through mist/ fog systems). Humidity is required for conditioning tissue culture plants after taken out from rooting media and transfer to pots under Green House.

List of instruments for Tissue culture :

1. **Atomizer :** Used for spray spirit in inoculation chamber
2. **Balances :** Weight chemicals for media preparation
3. **Microscope :** For observing cells and tissues
4. **Deep Freezer :** Storage of stock solutions, enzymes etc.
5. **Hot plate-cum-magnetic stirrer** : To dissolvechemicals

6. **Instrument's stand :** To keep sterilized instruments
7. **Low speed centrifuge :** To sediment cells and cleaning of protoplasts
8. **Metal trays and carts :** To transport culture flasks, racks of tubes etc.
9. **Oven :** For dry heat sterilization of glasswares
10. **pH meter :** To adjust pH of solution and media
11. **Autoclave :** For steam sterilization of media and apparatus
12. **Refrigerator :** For storage of chemicals, stock solutions and plant materials
13. **Shaker :** used for growing suspension culture
14. **Steamer :** To dissolve agar and melt media
15. **Laminar Air flow :** Used for aseptic manipulations of plant cells
16. **Water distillation apparatus or demineralization apparatus :** For double distilled or deionized water.

General Rules for Plant Tissue Culture Laboratory

1. Laboratory should have upto date records of all instruments and their working manuals and chemicals
2. All chemicals should be properly arranged and assigned specific area
3. Strong Acids and bases should stored separately
4. Volatile chemicals should be stored in fume hood
5. Do not drink, eat and smoke in the laboratory
6. Toxic chemicals must be handled precautionally
7. First aid kit should be placed in every working area
8. Fire extinguishers should be provided in each laboratory.

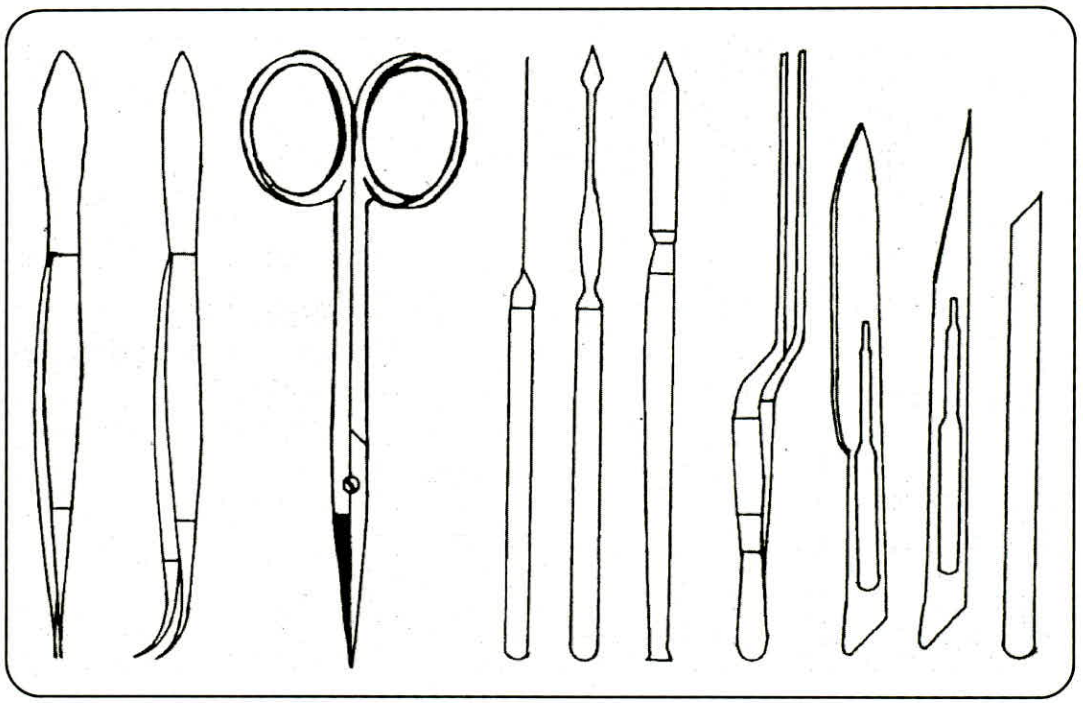


Fig. 1.1. A set of instruments used for Tissue culture work.

EXPERIMENT - 2

Aim : To study about techniques in plant tissue culture.

The ability to regenerate plants from single cell is important for progress with gene transfer in plants. Cell culture techniques are important for the regeneration of plants.

The vegetative propagation of stem cuttings or other growing plant parts to produce a genetic clones is common for some agri-horticultural crops. Potatoes, sugarcane, bananas etc. are cultivated by vegetative propagation. Techniques exist to propagate and regenerate whole plants from tissues, isolated plant cells or even protoplast culture. Cell culture technique have taken on added importance as biotechnology has progressed. Genetic engineering requires an ability to manipulate individual cells as recipients to isolate genes. Cell culture technique allows scientists to maintain and grow cells outside the organism and thus expand their ability to perform gene transfer and study the results. In addition cell culture allows scientists to regenerate numerous copies (clones) of manipulated varieties. The third use of cell culture is to regenerated somaclonal variants, plants with altered genetic traits that can prove useful as new or improved crops.

The important tissue culture techniques are :

i. Cell culture
ii. Callus culture
iii. Protoplast culture and micropropagation
iv. Anther culture/ haploid production
v. Somatic hybrids and cybrids
vi. Somatic embryogenesis
vii. Synthetic seed
viii. Germplasm conservation (gene bank)

General steps for Tissue culture Techniques

1. **Collection of explants materials :** The pieces of seedlings, buds, stem or storage organs, leaf materials and for cereals, immature embryos or basal stem sections of young plants are collected and stored in a screw cap bottle.
2. **Sterilization of the materials :** It is done by submerging in a dilute solution of the surface sterilants like calcium or sodium hypochlorite, hydrogen peroxide, bromine water, silver nitrate
3. **Removal of the sterilants :** it is done by rinsing thoroughly in distilled water.
4. **Transfer :** Transferring of the material to a sterile petri dish
5. **Preparation of suitable explants :** Prepared from surface sterilized materials using sterilized instruments.
6. Transferring the inoculums onto a suitable medium.
7. **Incubation :** at 26-28^{0}C in dark (low light) for 3-4 weeks for development of callus.
8. **Transfer of** callus in to a liquid medium
9. **Incubation** of flasks on a shaker at 150 rpm in continuous light at 26^{0}C for 4-6 weeks by decanting and replacing the volume with fresh medium at two weeks interval.
10. **Regeneration** of plants from cell suspension culture.

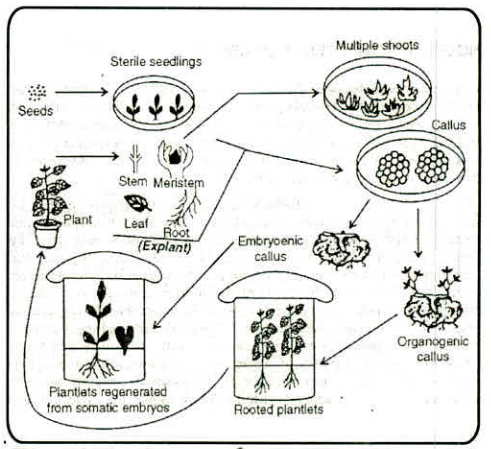


Fig. 2.1. General steps used for plant tissue culture.

EXPERIMENT - 3

AIM : Preparation of stock solutions of MS (Murashige & Skoog, 1962) basal medium and plant growth regulator stocks.

PRINCIPLE : The basal medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue. The tissue culture medium consists of 95% water, macro- and micronutrients, vitamins, aminoacids, sugars. The nutrients in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reactions. The macronutrients are required in millimolar (mM) quantities while micronutrients are needed in much lower (micromolar, μM) concentrations. Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions. Sugar is essential for in vitro growth and development as most plant cultures are unable to photosynthesize effectively for a variety of reasons. Murashige & Skoog (1962) medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration.

Plant growth regulators (PGRs) at a very low concentration (0.1 to 100 μM) regulate the initiation and development of shoots and roots on explants on semisolid or in liquid medium cultures. The auxins and cytokinins are the two most important classes of PGRs used in tissue culture. The relative effects of auxin and cytokinin ratio determine the morphogenesis of cultured tissues.

UNITS FOR SOLUTION PREPARATION

The concentration of a particular substance in the media can be expressed in various units that are as follows :

Units in weight

It is represented as milligram per litre (mg/l)

10^{-6} = 1.0 mg/l or 1 part per million (ppm)

10^{-7} = 0.1 mg/l.

10^{-8} = 0.001 mg/l or 1 μg/l.

Molar concentration

(M) = the molecular weight in g/l

1 A molar solution (M) contains the same number of grams of substance as is given by molecular weight in total volume of one litre.

1 molar mM = the molecular weight in mg/l or 10^{-3} M

1 μM = the molecular weight in μg/l or 10-^{6}M or 10^{-3} mM.

Conversion from milli molar (mM) to mg/l

For example, molecular weight of auxin 2,4-D = 221.0

1M 2,4-D solution consists of 221.0 g per litre

1 mM 2,4-D solution consists of 0.221 g per litre = 221.0 mg per litre

1 μM 2,4-D solution consists of 0.000221 g/l = 0.221 mg/l

Conversion from mg/l to mM

The molecular weight of $CaCl_2 - 2H_2O$

$= 40.08 + 2 \times 35.453 + 4 \times 1.008 + 2 \times 16 = 147.018$

(the atomic weights of Ca, Cl, H and O being 40.08, 35.453, 1.008 and 16.0 respectively).

If, 440 mg/l of $CaCl_2 - 2H_2O$ is to be converted into mM; then

$$\text{The number of mM } CaCl_2 . 2H_2O = \frac{\text{No. of mg } CaCl_2 - 2H_2O}{\text{Molecular weight of } CaCl_2 . 2H2O}$$

$$= \frac{440}{147.019} = 2.99 \text{ mM}$$

Thus, 440 mg/l $CaCl_2 - 2H_2O = 2.99$ mM

MATERIALS :

Amber bottles, Plastic beakers (100 ml, 500 ml and 1000 ml), Measuring cylinders (500 ml), Glass beakers (50 ml), Disposable syringes (5 ml), Disposable syringe, filter (0.22 µm), Autoclaved eppendorf tubes (2 ml), Eppendorf stand, Benzyl-aminopurine, Naphthalene acetic acid, Reagents.

MS NUTRIENTS STOCKS :

Nutrient salts and vitamins are prepared as stock solutions (20X or 200X concentration of that required in the medium) as specified. The stocks are stored at 4^0 C. The desired amount of concentrated stocks is mixed to prepare 1 liter of medium.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497.

MS major salts	**mg/1 L medium**	**500 ml stock (20X)**
1. NH_4NO_3	1650 mg	16.5 gm
2. KNO_3	1900 mg	19 gm
3. $Cacl_2 2H_2O$	440 mg	4.4 gm
4. $MgSO_4 7H_2O$	370 mg	3.7 gm
5. KH_2PO_4	170 mg	1.7 gm
MS minor salts	**mg/1 L medium**	**500 ml stock (200X)**
1. H_3BO_3	6.2 mg	620 mg
2. $MnSO_4 4H_2O$	22.3 mg	2230 mg
3. $ZnSO_4 4H_2O$	8.6 mg	860 mg
4. KI	0.83 mg	83 mg
5. $Na\ MoO_4 2H_2O$	0.25 mg	25 mg
6. $CoCL_2 6H_2O$	0.025 mg	2.5 mg

7. $CuSO_4 5H_2O$	0.025 mg	2.5 mg
MS Vitamins	**mg/1 L medium**	**500 ml stock (200X)**
1. Thiamine (HCl)	0.1 mg	10 mg
2. Niacine	0.5 mg	50 mg
3. Glycine	2.0 mg	200 mg
4. Pyrodoxine (HCl)	0.5 mg	50 mg
Iron, 500ml Stock (200X)		
Dissolve 3.725gm of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) in 250ml dH_2O. Dissolve 2.785gm of $FeSO_4.7H_2O$ in 250 ml dH_2O. Boil Na_2EDTA solution and add to it, $FeSO_4$ solution gently by stirring.		

PLANT GROWTH REGULATOR STOCK

The heat-labile plant growth regulators are filtered through a bacteria-proof membrane (0.22 μm) filter and added to the autoclaved medium after it has cooled enough (less than 60^0C). The stocks of plant growth regulators are prepared as mentioned below.

Plant Growth Regulator	Nature	Mol. Wt.	Stock (1 mM)	Soluble in
Benzyl aminopurine	Autoclavable	225.2	mg/ ml	1N NaOH
Naphtalene acetic acid	Heat labile	186.2	mg/ ml	Ethanol

The desired amount of plant growth regulators is dissolved as above and the volume is raised with double distilled water. The solutions are passed through disposable syringe filter (0.22 ?m). The stocks are stored at -200 C.

EXPERIMENT - 4

AIM : Sterilization techniques for establishment and maintenance of cultures.

PRINCIPLE :

Maintenance of aseptic environment :

All culture vessels, media and instruments used in handling tissues as well as the explants must be sterilized. The importance is to keep the air surface and floor free of dust. All operations are carried out in laminar air-flow, a sterile cabinet. Infection can be classified in three ways:

1. The air contains a large quantity of suspended microorganisms in the form of fungal and bacterial spores.
2. The plant tissue is covered with pathogens on its surface.
3. The human body (a skin, breathe etc) carries several microorganisms.

In general, the methods of elimination of these sources of infection can be grouped under different categories of sterilization procedures:

1. Preparation of sterile media, culture vessels and instruments (sterilization is done in autoclave)
2. Preparation of sterile plant growth regulators stocks (by filter sterilization)
3. Aseptic working condition
4. Explants (isolated tissues) are sterilized using chemical sterilents, e.g. $HgCl_2$ and NaOCl.

Sterilization : It follows that all the articles used in the plant cell culture must be sterilized to kill the microorganisms that are present.

A. Steam or Wet sterilization (Autoclaving) : This relies on the sterilization effect of super-heated steam under pressure as in a domestic pressure cooker. The size of the equipment used can be as small as one litre or even as large as several thousand litres. Most instruments/ nutrient media are sterilized with the use of an autoclave and the autoclave has a temperature range of 115- 135^{0}C. The standard conditions for autoclaving has a temperature of 121^{0}C and a pressure of 15 psi (Pounds per square inch) for 15 minutes to achieve sterility. This figure is based on the conditions necessary to kill thermophilic microorganisms. The time taken for liquids to reach this temperature depends on their volume. It may also depend on the thickness of the vessel. The temperature of 121^{0}C can only be achieved at 15 psi. The efficiency of autoclave can be checked in several ways :

The most efficient way is to use an autoclave tape. When the autoclave tape is autoclaved, a reaction causes dark diagonal strips to appear on the tape indicating that it is autoclaved.

Precautions :

1. Excessive autoclaving should be avoided as it will degrade some medium components, particularly sucrose and agar breakdown under prolonged heating. Especially when under pressure and in an acidic environment. A few extremely thermoduraic microorganisms exist that can survive elevated temperature for sometime. But 15-30 minutes kill even those.
2. At the bottom of the autoclave the level of water should be verified.

3. To ensure that the lid of the autoclave is properly closed.
4. To ensure that the air- exhaust is functioning normally.
5. Not to accelerate the reduction of pressure after the required time of autoclaving. If the temperature is not reduced slowly, the media begin to boil again. Also the medium in the containers might burst out from their closures because of the fast and forced release of pressure.
6. Bottles, when being autoclaved, should not be tightly screwed and their tops should be loose. After autoclaving these bottles are kept in the laminar air-flow and the tops of these bottles are tightened on cooling.

B. Filter sterilization : Some growth regulators like amino acids and vitamins are heat labile and get destroyed on autoclaving with the rest of the nutrient medium. Therefore, it is sterilized by filtration through a sieve or a filtration assembly using filter membranes of 0.22 μm to 0.45μm size.

C. Irradiation : It can only be carried out under condition where UV radiation is available. Consequently, its use is restricted generally to purchased consumables like petridishes and pipettes. UV lights may be used to kill organisms in rooms or areas of work benches in which manipulation of cultures is carried out. It is however, dangerous and should not be turned on while any other work is in progress. UV light of some wavelengths can damage eyes and skin.

D. Laminar Airflow Cabinet : This is the primary equipment used for aseptic manipulation. This cabinet should be used for horizontal air-flow from the back to the front, and equipped with gas corks in the presence of gas burners. Air is drawn in electric fans and passed through the coarse filter and then through the fine bacterial filter (HEPA). HEPA or High Efficiency Particulate Air Filter is an apparatus designed such that the air-flow through the working place flows in direct lines (i.e. laminar flow). Care is taken not to disturb this flow too much by vigorous movements. Before commencing any experiment it is desirable to clean the working surface with 70% alcohol. The air filters should be cleaned and changed periodically.

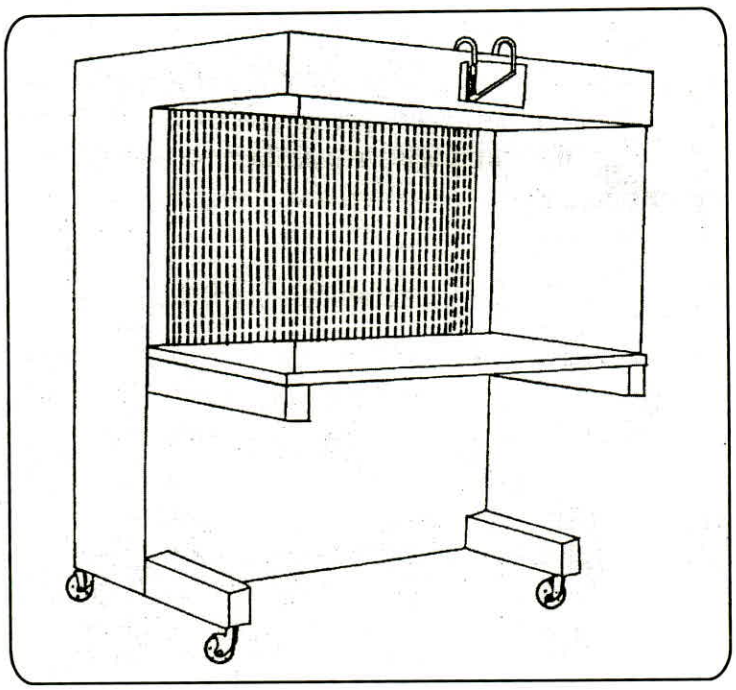


Fig.4.1. A laminar flow chamber

EXPERIMENT - 5

Aim : To study about aseptic manipulation of various explants.

The surface of plant parts are infected with a wide range of microbial contaminants like fungi, bacteria, virus etc. They have adverse effect on growth and development of culture. Therefore, the explants should be sterilized with disinfectant solutions before planting these in the nutrient medium. The important surface sterilants are as below :

Disinfectant	Exposure time (min)	Effectiveness	Conc. (%) used
Antibiotics	30-60	Fairly good	4-50 mg/l
Bromine water	2-10	Very good	1-2
Calcium hypochlorite	5-30	Very good	9-10
Ethyl alcohol	0.1-5.0	Good	70-95
Hydrogen peroxide	5-15	Good	3-12
Silver nitrate	5-30	Good	1.0
Sodium hypochlorite	5-30	Very good	0.5-1.5

Requirements : Explant materials, commercial bleach/calcium hypochlorite, ethanol (70%), Surfactant (Triton-X/ tween 20 or 80), mold detergent, screw cap bottles, sterile forceps, scalpels and sterile distilled water.

Procedure :

1. Make small pieces of explants materials (seedlings, swelling buds, stem or storage organs, leaf materials) using scalpel.
2. Wash explants in a mild detergent.
3. Rinse explants under running tap water for 10-30 min.
4. Rinse explants in 70 % ethanolfor30 seconds and material left exposed in the sterile hood for evaporation of alcohol.
5. Aseptically transfer explants into a vial containing wetting agent (surfectant) to reduce surface tension and for better surface contact and 20 % commercial bleach (5 % sodium hypochlorite), a disinfectant.
6. Keep the explants submerged in the above solution for 5-30 min., shaking the vial 2-3 times, for sterilization.
7. Decant the liquid
8. Pour an adequate amount of sterile distilled water into the vial and replace the cap.
9. Shake the vial, a few times and discard the water.
10. Repeat steps 9 and 10 to rinse explants 4-5 times.

EXPERIMENT - 6

AIM : Callus induction and Plant Regeneration.

Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants in vitro from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.

Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Callus may be serially subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are favoured by the medium and come to dominate the culture.

Callus tissue from different plants species may be different in structure and growth habit: white or coloured, soft (watery) or hard, friable (easy to separate in to cells) or compact. The callus growth within a plant species is dependent on various factors such as the original position of the explant within the plant, and the growth conditions.

Although the callus remains unorganized, with increasing growth, some kinds of specialized cells may be formed again. Such differentiation can appear to take place at random, but may be associated with centers of morphogenesis, which can give rise to organs such as roots, shoots and embryos.

AIM : To induce callus from the explants of ***Phaseolus mungo (Green Gram)***

Reagents and other requirements

1. Culture tubes or conical flasks containing media
2. Sterile Petri dishes
3. Scalpel, blades, forceps and steel dissecting needles
4. Sterile distilled water
5. Alcohol
6. Detergent (Tween 20, Teepol, etc.)
7. Sterilants – $HgCl_2$, Sodium. Hypochlorite
8. Nutrition medium reagents – MS basic salts and vitamins
9. Growth regulators – 2, 4-D

Plant material – Green gram

Media : Seed Germination: MS Medium
Callus Induction: MS + 2, 4-D (2mg/lL)

Procedure :

1. Aseptically-germinated seedlings will be free of contaminants. This can be directly used as an explant.
2. Transfer the plant from culture tube to sterile Petri plate containing filter paper.
3. Excise root, internode, hypocotyle, leaf and cotyledon and transfer in to another sterile Petri plate.
4. Each explant is separately transferred into appropriate concentration of MS medium with sterile forceps and culture tube is closed with aluminium foil.
5. Culture tubes are incubated at 25 ± 2^0C with a photoperiod of 16 hours light and 8 hours dark and observations are taken every week.

Result : The undifferentiated mass of cells was formed from the inoculated leaf explant.

EXPERIMENT - 7

AIM : Micropropagation of Tobacco plant by leaf disc culture.

PRINCIPLE : Plant cells and tissues are totipotent in nature i.e., every individual plant cell or tissue has the same genetic makeup and capable of developing along a "programmed" pathway leading to the formation of an entire plant that is identical to the plant from which it was derived. The totipotency of the plant cells and tissues form the basis for in vitro cloning i.e., generation or multiplication of genetically identical plants in in vitro culture. The ability to propagate new plants from a cells or tissues of parent plant has many interesting possibilities.

Micropropagation is used commercially to asexually propagate plants. Using micropropagation, millions of new plants can be derived from a single plant. This **rapid multiplication** allows breeders and growers to introduce new cultivars much earlier than they could by using conventional propagation techniques, such as cuttings. Micropropagation also can be used to establish and maintain **virus-free plant stock**. This is done by culturing the plant's apical meristem, which typically is not virus-infected, even though the remainder of the plant may be. Once new plants are developed from the apical meristem, they can be maintained and sold as virus-free plants.

Micropropagation differs from all other conventional propagation methods in that aseptic conditions are essential to achieve success. The process of micropropagation can be divided into four stages :

1. **Initiation stage :** A piece of plant tissue (called an explant) is (a) cut from the plant, (b) disinfested (removal of surface contaminants), and (c) placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.
2. **Multiplication stage :** A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. A cytokinin is a plant growth regulator that promotes shoot formation from growing plant cells.
3. **Rooting or preplant stage :** Growing shoots can be induced to produce adventitious roots by including an auxin in the medium. Auxins are plant growth regulators that promote root formation. For easily rooted plants, an auxin is usually not necessary and many commercial labs will skip this step.
4. **Acclimatization :** A growing, rooted shoot can be removed from tissue culture and placed in soil. When this is done, the humidity must be gradually reduced over time because tissue-cultured plants are extremely susceptible to wilting.

Micropropagation has become more feasible with the development of growth media that contain nutrients for the developing tissues. These media have been developed in response to the needs of plant species to be multiplied. This laboratory exercise will use a growth medium (MS) that will contain the macronutrients, micronutrients, vitamins, iron and sucrose. A combination of cytokinin (BAP) and auxin (NAA) will be supplemented to basal medium (MS) for induction of multiple shoots from the leaf disc explant.

MATERIALS REQUIRED : Beakers, Measuring cylinders, Conical flasks, Cotton plugs, Myoinositol, Sucrose, BAP (1mM stock), Agar Agar, Forceps, Blade Holder (No.3), Sterilzed blades (No.11), NAA (1 mM stock), Micropipettes, sterilized microtips, cork borers, petridishes.

INSTRUCTIONS : The shoot multiplication medium for tobacco leaf disc is MS basal + BAP (2.5 μM) + NAA (0.5 μM)

Preparation of MS medium (1000 ml)

- MS Major (20X) 50 ml
- MS Minor (200X) 5 ml
- MS Vitamin (200X) 5 ml
- Iron (200X) 5 ml
- Myoinositol 100 mg
- Sucrose 30 gm (3%)
  - → Add BAP at this stage (Calculate, how much to add?)
  - → Make final volume to 1000 ml by double distilled water
  - → Set pH at 5.8
  - → Add agar agar 8 gm/L (0.8%), melt the agar agar in microwave oven
  - → Sterilize the media at 15 psi/121^{0}C for 15 minutes
  - → After autoclaving, gently swirl the medium to mix the agar. When the agar is completely dissolved and mixed, the medium should appear clear and not turbid.
  - → Add filter sterilized NAA (desired amount, calculate?) once the temperature of the medium cools down to 60^{0}C.

Cut the tobacco leaf into discs and culture tobacco leaf disc in the medium. Maintain the cultures under cool white fluorescent light in a 16 h photoperiod regime at 25±2^{0}C. Observe the cultures periodically.

MAJOR STAGES OF MICROPROPAGATION

↓

Selection of an elite mother plant

↓

Explant pieces

↓

Trimming

↓

Surface sterilization

↓

Washing

↓

Establishment of cultures on appropriate growth medium

↓

Transfer of multiplication medium

↓

Rapid shoot or embryo formation

↓

Embryo shoots or plantlets transferred to sterilized soil or artificial medium by different gradual weaning processes

EXPERIMENT - 8

AIM : Micropropagation of Rice by indirect organogenesis from embryo.

PRINCIPLE : The regeneration of plants through an intermediate callus phase is termed as "Indirect regeneration". The explants (meristematic tissue) dedifferentiate to form callus, an unorganized growth of dedifferentiated cells. Group of cells in callus reorganize to from meristemoid, similar to meristem tissue. Meristemoid redifferentiate to form shoot buds, which finally regenerate to plantlets. This experiment will use a growth medium (MS) supplemented with 2,4-D (auxin) to induce callus. The whitish-friable calli will be selected for redifferentiation on MS medium containing the BAP (cytokinin). The healthy-growing calli with green spots will be subcultured on the fresh medium. The regenerating shoots will be transferred to basal medium for root induction.

MATERIALS : Plastiware and glassware for medium preparation, MS stocks, 2,4-D, casein hydrolysate, culture vessels and rice seeds.

Callus induction medium from rice seeds : MS or N6 basal + 2,4-D (2.0 mg/L) + Casein hydrolysate (0.3 1.0 mg/L)

Redifferentiation medium : MS basal + BAP (3 mg/L) **Rooting medium :** MS basal.

A. Preparation of callus induction media

The carbon source in callus induction medium can be maltose or sucrose (30 g/L), and casein hydrolysate is used as an optional supplement. The concentrations are optimized for each variety. Usually, MS is used for rice var. Indicas and N6 for Japonica.

- Mix all the ingredients together (i.e. basal salt, carbon source, vitamins, hormones, etc.) in 700 ml ddH_2O. Stir it until all they dissolve.
- Make final volume to 1000 ml by ddH_2O.
- Adjust the pH to 5.8, add agar agar and autoclave for 15 min.
- Dispense the media to sterile petridishes (20-25 ml each) inside laminar hood. Allow them to cool.

B. Dehulling, sterilization and plating of seeds

- Remove carefully the lemma and palea using forceps, avoiding any damage to the embryo.
- After dehulling, select the healthy and shiny seeds. Place them in a sterile flask and surface sterilize with 70% ethanol for 1-2 minutes. Rinse 3 times with sterile dH_2O.
- Sterilize the seeds again in 50% Chlorox (Zonrox-a commercial bleach) for 25-30 minutes, preferably under vacuum or in a shaker. (A drop of Tween 20 or any surfactant can be added to enhance the effect of chlorox.
- Rinse 3-5 times with sterile dH_2O to remove all of the chlorox. Place the seeds in sterilized filter paper for drying before plating.
- Put 10-15 seeds in each sterile petridish containing 30 ml of solidified callus induction medium and incubate them in the dark room for 30-40 days. Check the culture for contamination 3 days after inoculation, and every week thereafter.

C. Selecting calli for organogenesis

- Select the embryogenic calli (whitish, globular, friable, dry, free of any differentiated structures such as root-like or shoot-like appearance).
- Transfer the healthy and growing embryogenic calli into MS regeneration media containing 3 mg/L BAP.

D. Regeneration and rooting

- Transfer the healthy and growing embryogenic calli into MS regeneration media containing 3 mg/L BAP.
- Subculture the healthy and proliferating calli with green spots into culture bottles containing fresh regeneration media with same concentration of BAP.
- After one month, transfer the proliferated shoots (3-4 cm) to rooting media free or devoid of any hormone.
- Establish the rooted plantlets in pot containing soil.

EXPERIMENT - 9

Aim : To isolate embryos of Cicer aertinum and perform in vitro culture.

Requirement :

1 Sterilants - alcohol, HgCl2, sodium hypochlorite.
2. Nutrition medium reagents - MS basic salts and vitamins.
3. Growth regulators – usually not required for embryogenesis.
4. Plant Material- Embryo of Cicer auritinum.
5. Culture tubes containing media.
6. Sterile Petri dishes.
7. Scalpel, blades, forceps knives and steel-dissecting needles.
8. Sterile distilled water.

Procedure :

1. The seeds were washed by submerging them in water with a few drops of detergent in a beaker and shake them by hand.
2. Excise the embryo and collected without any damage.
3. It was washed with distilled water and then treated with 70% alcohol for 30 seconds.
4. This was followed by rinsing completely with distilled water and then transferred to 20% sodium hypochlorite, where it was left for 0 minutes.
5. Then the embryo was thoroughly rinsed with distilled water for 3 times and dried using the autoclaved tissue paper and inoculated in the culture tubes containing the MS medium.
6. The culture tubes were incubated at 25°C under 16 h photoperiod for 2 to 3 weeks.

Result :

The plant was developed from inoculated embryo.

EXPERIMENT - 10

AIM : To isolate and inoculate anthers for haploid production.

PRINCIPLE :

Haploids refer to those plants which possess a gametophytic number of chromosomes in their sporophytes. Haploids may be grouped into two broad categories:

(a) monoploids which possess half the number of chromosomes from a diploid species.

(b) Polyhaploids which possess half the number of chromosomes from a polyploidy species.

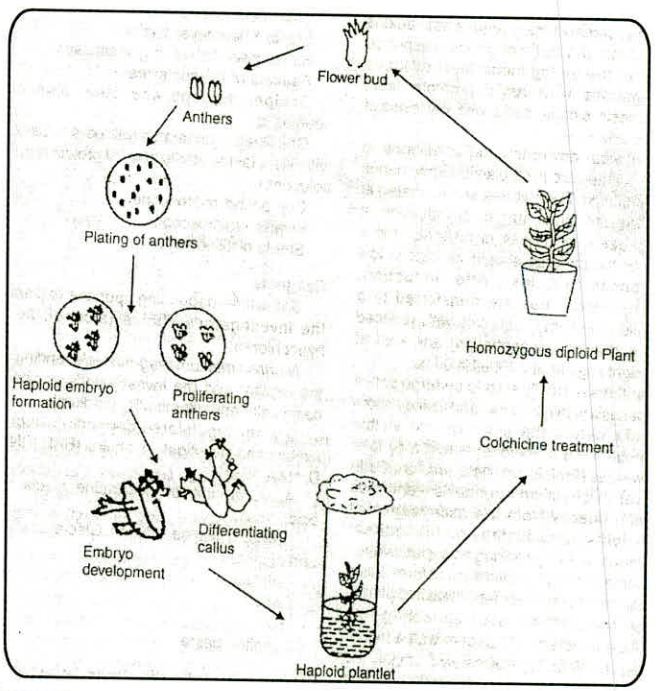
Haploid production through anther culture has been referred to as androgenesis while gynogenesis is the production of haploid plants from ovary or ovule culture where the female gamete or gametophyte is triggered to sporophytic development.

MATERIALS REQUIRED :

Anthers from Hibiscus, MS medium, growth factors, 70% ethanol, 2% mercuric chloride, Meso inositol, Scissors, Scalples, Petriplates, Forceps.

PROCEDURE :

1. Flower buds of Hibiscus were collected.
2. The flower buds are surface sterilized by immersing in 70% ethanol for 60 sec followed by immersing in 2% sodium hypochloride solution for 1 min or in mercuric chloride.
3. The buds were washed four or five times with sterile distilled water.
4. The buds were transferred to a sterile Petridish.
5. The buds were split open using a blade and the anthers were removed without damage and the filaments were removed.
6. The anthers were placed horizontally on the MS medium supplemented with different concentration of plant growth regulators or mesoinositol.
7. The Petriplates were sealed and incubated in dark at 28°C.
8. The Petriplates were examined for the germination of anthers.



RESULT :

The anther underwent germination leading to the formation of haploid plantlets.

EXPERIMENT - 11

AIM : To study about hardening and acclimatization of regenerated plants.

Plants developed in vitro condition and growing under natural conditions have several morphological and physiological distinctions. in-vitro developed plants are adopted to high humidity, optimal nutrient supply, low light intensity and good supply of sucrose conditions. Leaves produced under such condition have thinner cuticular waxy coatings and more open mesophyll tissues. These also have reduced chlorophyll because photosynthesis process are inhibited by sucrose in the medium. Gradual exposure to normal conditions produce a progress in morphological and physiological adaptation, also referred as 'hardening off'.

The relative humidity in vitro may be reduced by loosening the vessel and increasing light intensity in the process for transplanting that ultimately activates chlorophyll synthesis and photosynthetic activity.

1. Gently remove the well-rooted plantlets from culture vessel, keeping the roots intact. Carefully rinse the roots with lukewarm water to remove the agar attached to it.
2. Plant the regenerated plantlets in small plastic pots with sterile soil mix comprising sand: FYM: soil in 1:1:1 ratio or peat: vermiculite: sand in 1:1:1 proportion. Make sure the soil is moist with water. After planting put plastic cover or inverted plastic vessel/ beaker to maintain high humidity.
3. Keep the pots in high humidity chambers/ mist, fog chambers under 25 ^{0}C in diffused light. Open inverted cover to allow free exchange of air briefly after 1 week for 1 hr.. After 2nd week gradually increase the exposure to air for several hours per day. After 2-3 weeks remove the cover to adjust to natural environmental conditions. Fungicide or insecticides should not be sprayed during acclimatization and diseased or infected plants should be removed quickly.

EXPERIMENT - 12

AIM : To study about somatic embryogenesis.

The embryos regenerate from somatic cells, tissues or organs either de novo or directly from the tissues (adventives origin), which is opposite of zygotic or sexual embryogenesis. Induction of somatic embryogenesis requires a single hormonal signal to induce a bipolar structure capable of forming a complete plant.

Somatic embryogenesis can be induced either directly from the explants tissue in the absence of callus formation or via the callus from the explants. Somatic embryogenesis encompasses various stages from callus initiation to embryo development and maturation and subsequently, plantlet formation.

Requirements : All instruments for basic plant tissue culture laboratory and stereo zoom microscope, Sterilants (alcohol, $HgCl_2$, Sod. hypochlorite), MS basic Nutrient media, vitamins, Growth regulator (2,4-D).

Plant material : Hypocotyle/ petiole of carrot.

Steps :

1. Wash the seeds by submerging in water with detergent in a beaker and shake them.
2. Submerge the seeds in 70% alcohol for 30-60 sec., decant the alcohol.
3. Transfer the seeds to a flask or beaker containing 20-40% commercial Sod. hypochlorite solution for 15-20 min. or 0.01 to 0.1% $HgCl_2$ for 5-10 min. Rinse them five times with sterile distilled water.
4. Place the 2-3 seeds/ culture vessel on the surface of MSO agar medium.
5. Incubate the cultures at 25^0C under 1000 lux light intensity for 1-2 weeks.
6. Collect the germinated seedlings when the cotyledons are fully expanded. Place each seedlings on a sterile petri dish.
7. Place the hypocotyle/ petiole section on the MSE and MS+(0.1-2.0) mg/L 2,4-D.
8. Incubate the culture in dark at 25^0C for 4-8 weeks
9. maintain the callus by subculturing small pieces on fresh medium every 3-4 weeks. callus will contain pro-embryo initial cells.
10. Place 0.5-1 cm^2 callus pieces on MSO agar media without growth regulators and incubate the cultures at 25^0C and 16 h. photoperiod with 1000 lux light intensity. Within 2-3 weeks culture will exhibit embryos and green plantlets.
11. Take out individual or group of plantlets from the callus mass and transfer on half strength MS medium under 16h. photoperiod with high light intensity of ~5 klux with 4-5 weeks culture will resemble seedling of carrot.
12. Transfer the plantlets to small pots containing sterile peat moss and vermiculite in 1:1 ratio. Enclose the plantlets with plastic containet to maintain high humidity.

    Transfer the plants to the soil and follow the procedure of plant establishment and hardening.

EXPERIMENT -13

SYNTHETIC SEED PREPARATION

Aim : To prepare hydrated synthetic seeds in vitro

Materials Required :

Beaker
Petri dish
Micropipette
Microtips

Chemicals :

Sodium alginate (3%)
Calcium chloride (4%)
MS Medium
Distilled water

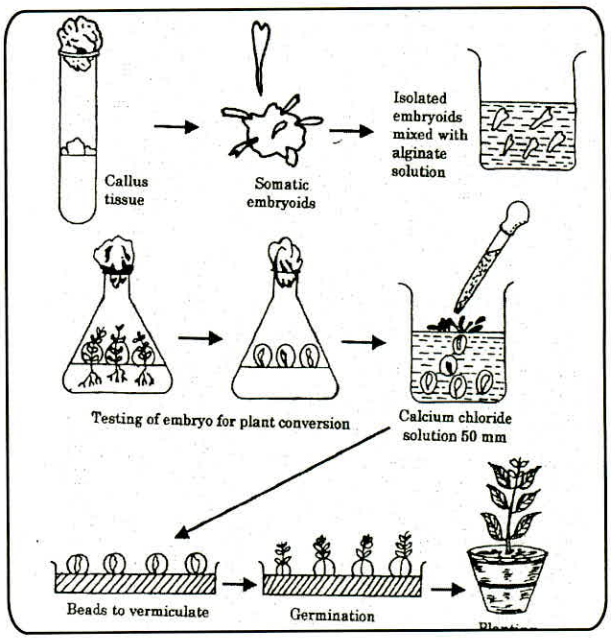


Fig.13.1. Process of Artificial seed production

Procedure :

1. MS Medium is prepared by adding macro and micro-nutrients.
2. Add 3 % sodium alginate (hydrogel) instead of agar-agar.
3. Prepare calcium chloride solution.
4. Dispense both sodium alginate + MS medium and calcium chloride in separate flasks.
5. Axillary buds with nodal portion (0.5 cm length) or shoot tips from regenerated shoots or somatic embryos from cultured callus.
6. Nodal portions or shoot tips or somatic embryos are blotted to dry on sterile filter paper.
7. These explants are then mixed in 3 % sodium alginate + MS medium supplemented with 2 % sugar.
8. After thorough mixing the explants alongwith the medium containing sodium alginate are taken in ink fillers or broad mouthed pipettes and slowly dropped into a sterile solution of CaCl2. H2O (3.675 g/ 500 ml) and allowed to form beads.
9. They are retained in this complexing agent for 30 minutes and formed encapsulated beads.
10. The alginate beads are then collected and transferred to sterile filter paper placed in Petri dishes.
11. The blot dried synthetic seeds are stored for 1-6 months at 40C.

Result :

Hydrated or encapsulated seeds are formed.

EXPERIMENT -14

ISOLATION OF PROTOPLASTS

Protoplast is the living material of the cell where as an isolated protoplast is the cell from which the cell wall is removed. In plant breeding programme many desirable combination of characters could not be transmitted through the conventional method of genetic manipulation. higher plants that could lead to the genetic process involving fusion between the subsequent developments of a product to a hybrid plant is known as somatic hybridization.

Plant protoplasts can be isolated from cells by two methods :

1. Mechanical method
2. Enzymatic method.

MECHANICAL METHOD

Aim : To isolate protoplast by mechanical method

Principle :

Protoplast can be isolated from almost all plant parts: roots, leaves, fruits, tuber, root nodules, pollen mother cell etc. Protoplast isolated by mechanical is a crude and tedious procedure. Cells are plasmolysed causing the protoplast to shrink from the cell wall. The protoplast obtained from this method is then cultured on suitable culture medium. The principle deficiency of this approach is that the protoplast released is few in number. Mechanical isolation was that of only historical event now.

Materials Required :

1. Plant leaves – Duranta repens, Mortar and pestle, Phosphate buffer pH-7.0, 0.3 M sorbitol, 0.3 M mannitol, Glass slides, Microscope.

Procedure :

1. Young leaves were obtained from plants growing out doors and initially washed with tap water to remove any dust particles.
2. The leaves were washed with phosphate buffer and homogenized gently with the mortar and pestle.
3. The crude protoplast suspension was centrifuged at very low 50-100 rpm for 10 minutes.=
4. The supernatant containing intact protoplast was carefully pipetted out and the pellet containing cell debris and other cell organelles were discarded.
5. Small volume of supernatant was placed in the slides and covered with coverslip.
6. The slide was observed in light microscope to find out viable protoplast

Result : The spherical shaped protoplasts were observed using the microscope.

AIM : To isolate protoplasts by enzymatic method

PRINCIPLE :

Protoplasts are isolated by treating tissues with a mixture of cell wall degrading enzyme in solution, which contain osmotic stabilizer. A most suitable source of protoplasts is mesophyll tissue from fully expanded leaves of young plants or new shoots. The release of protoplast is very much dependent on the nature and composition of enzymes used to digest the cell wall. There are three primary components of the cell wall which have been identified as cellulose, hemicellulase and pectin substance. Pectinase (macrozyme) mainly degrades the middle lamella while cellulose and hemicellulase degrades the cellulose and hemicellulosic components of the cell wall. During this enzymatic treatment, the protoplast obtained should be stabilized because the mechanical barrier of the cell wall which offered support has been broken.

For this reason an osmoticm is added which prevents the protoplast from bursting.

MATERIALS REQUIRED : Young leaves, 70% ethanol, 2% cellulose, 13% mannitol, 0.5% macrozyme, CPW salt solution:

KH2PO4 - 27.2mg/l,	KNO3 - 101mg/l
CaCl2 - 1480mg/l	MgSo4 - 246mg/l
KI - 0.16mg/l	CaSo4 - 0,026mg/l
pH - 5.8.	

PROCEDURE :

1. The young leaves were collected and washed in sterile distilled water thrice.
2. The leaves were cut into small bits.
3. Then the leaves were kept immersed in 13% mannitol for 1 h for pre-plasmolysis.
4. Mannitol was removed after incubation ant sterilized enzyme mixture (Cellulase + macerozyme) was added and incubated at 25°C in a shaker for 12 h.
5. The filtrate was centrifuged at 100g for 5 min to sediment the protoplast.
6. The supernatant was removed and the protoplast pellet was suspended in 10ml of CPW +21% sucrose solution.
7. The mixture was centrifuged at 100g for 5 min. The viable protoplast will float to the surface of the sucrose solution.
8. The supernatant was collected and viewed under microscope.
9. The protoplasts were visualized in microscope.

RESULT : Protoplasts were isolated by enzymatic method and viewed under the microscope.

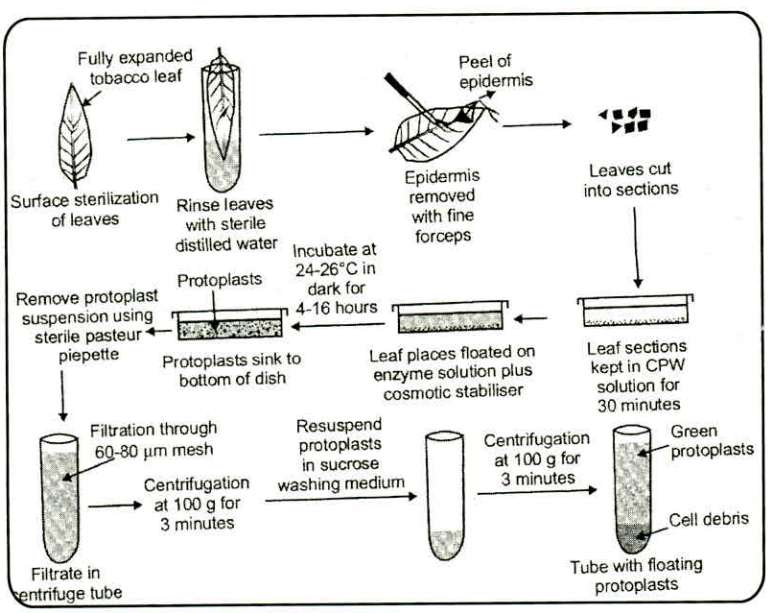


Fig. 14.1. Steps of protoplast isolation

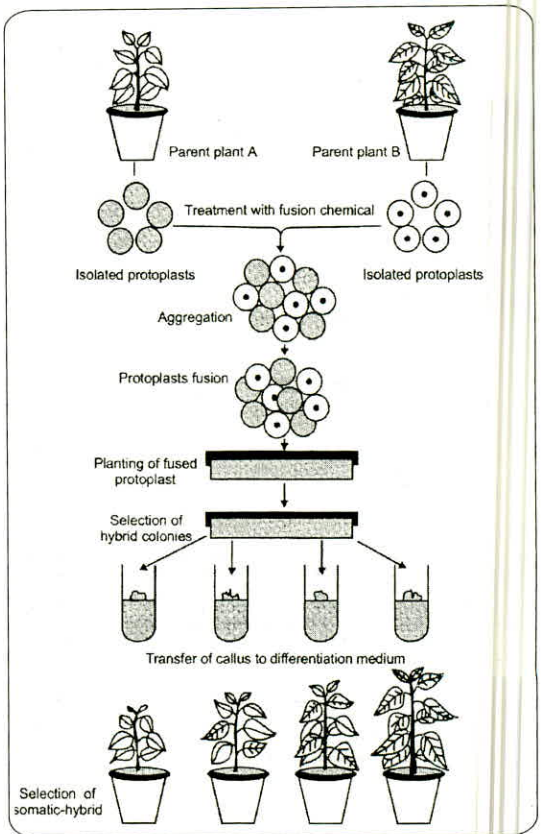


Fig. 14.2. Protocol of Protoplast fusion

EXPERIMENT - 15

AIM : Isolation of plant genomic DNA by modified CTAB method.

PRINCIPLE : The DNA to be use for molecular analysis usually requires that it is isolated and purified. Extraction of high molecular weight of DNA free from protein and RNA is essential for all molecular biology works. The extraction of genomic DNA from plant material requires cell lysis, inactivation of cellular nucleases and separation of the desired genomic DNA from cellular debris. Ideal lysis procedure is rigorous enough to disrupt the complex starting material (plant tissue), yet gentle enough to preserve the target nucleic acid. The cetyl trimethyl ammonium bromide (CTAB) protocol (developed by Murray and Thompson in 1980) is appropriate for the extraction and purification of DNA from plants and plant derived foodstuff and is particularly suitable for the elimination of polysaccharides and polyphenolic compounds otherwise affecting the DNA purity and therefore quality.

Plant cells can be lysed with the ionic detergent CTAB, which forms an insoluble complex with nucleic acids in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. The DNA complex is solubilised by raising the salt concentration and precipitated with ethanol or isopropanol.

MATERIALS :

Plant samples (leaf, callus etc.), Liquid nitrogen, Sterile pestle and mortar, Sterile spatulas, Waterbath set at 65^0C, Sterile eppendorf tubes and desired reagents

REAGENTS :

2-Mercaptoetanol or β mercaptoethanol	Cetyl trimethyl ammonium bromide (CTAB)
Chloroform	Ethanol
Glacial acetic acid	HCl
Isoamyl alcohol	Isopropyl alcohol or isopropanol
Na_2-EDTA	NaCl
NaOH	Phenol
Potassium acetate	RNase A
Sodium acetate	Sodium dodecyl sulphate (SDS)
Tris base	

Stock Solution

DNA Extraction buffer

Triazma base	12.11 g
EDTA disodium salt	18.07 g
NaCl	29.22 g
SDS	12.05 g

*SDS to be added after autoclaving when the solution was hot. The pH was adjusted to 8.0 and final volume made to 1 litre.

5M Potassium acetate

490.7 g Pot. Acetate was dissolved in 800 ml of distilled water and thenn final volume make upto one litre and autoclaved.

3 M Sodium acetate

Dissolve 123.05 g of Sod. Acetate in 350 ml of distilled water and pH adjusted to 5.2 and final volume made upto 500 ml and autoclaved.

RNase A

Stock solutions

1. 10mM Tris HCl (pH 7.5)
2. 15mM NaCl

10 mg of RNase A was added per ml of above solution, mixed, boiled and allowed to cool at room temperature and stored in freezer.

1M Tris (pH 8.3 at 25^{0}C)

30.28 g of triazma dissolved in 200 ml of distilled water. The pH set to 8.3 using conc. HCL. The final vol. adjusted to 250 ml with distilled water and sterilized by autoclaving.

1M KCl

18.64 g of Potassim chloride was dissolved in 200 ml of water and final volume made to 250 ml and autoclaved.

Iso propanol (pre chilled), Absolute alcohol (pre chilled), 70 % Ethanol (pre chilled), Solutions for electrophoresis 50X TAE buffer

Traizma base	121.00 g
Glacial Acetic acid	28.55 ml
0.5M EDTA (pH 8.0)	100.00 ml
Distilled water	50.45 ml
Total	300.00 ml

Tank buffer (1X TAE)

20 ml 50X TAE+980 ml of distilled water (for 1 Lt. Tank buffer)

2X CTAB extraction buffer

5 g CTAB and 20.35 g NaCl were dissolved in 200 ml double distilled water. Later 25 ml 1M tris HCl and 10 ml 0.5 M EDTA were added and stirred vigorously on a magnetic stirrer. Volume was made upto 250 ml and stored at room temperature, 20 µl/ 20 ml 2- mercapto ethanol was added prior to use.

Extraction of Plant Genomic DNA using CTAB (Cetyl trimethyl ammonium bromide)

This method was given by Murray and Thomson (1980) Before starting add β mercapto ethanol to CTAB extraction buffer @ 20 µl / 20 ml. Then follow the protocols:

1. Take about 100 mg of tender leaf sample and grind in 400 µl 2X CTAB extraction buffer with a glass rod on spot plate.

2. 400 µl more of 2X CTAB extraction buffer added and mixed thoroughly. In ~700 µl of solution and then transferred in to 1.5 ml eppendorf tube. Or the cut leaf can be collected in 2 ml centrifuge tubes upto half mark followed by adding 800 µl CTAB buffer and crushing by tissue lyzer.
3. Incubate at 65°C on water bath for 15-20 min. and then cooled briefly and 700 µl of chloroform: Isoamyl alcohol (CIA-24:1) was added.
4. The contents are shaken by hand intermittently and kept at room temperature for 15 min. Tubes are centrifuged at 12,000 rpm for 20 min.
5. Give RNase treatment to the sample for purification.
6. 600 µl of upper aqueous phase containing DNA is transferred to a new 2 ml eppendorf tube. 900 µl of absolute ethanol was added and mixed gently and tubes are kept for 2 hrs at -20°C.
7. Then the sample was centrifuged for 3 min at 10,000 rpm, the supernatant was decanted. The pellet was washed with 70% etnanol.
8. DNA pellet was air drid and then dissolved in 50 µl of TE buffer.
9. Store in -20°C for after use.

Genomic DNA Extraction through Dellaporta et al., (1983) protocol

- Fresh leaves are cut into fine small pieces, and crushed in liquid nitrogen using mortar and pastle. The leaf powder (approx. 10 ml level) transferred immediately into 50 ml centrifuge tube.
- 15 ml of extraction buffer (preheated to 65°C and added with 3.8 g od sodium bisulphate per 1000 ml) was added in each tube and incubated at 65°C for 20 min using a water bath with occasional mixing.
- 5 ml of 5M potassium acetate added to each sample and mixed vigorously and the tubes were incubated on ice for 45 min with shaking.
- The samples are centrifuged for 15 min at 3500 rpm and then supernatant filtered through mira cloth in a new 50 ml centrifuge tube.
- 2/3 volume of pre chilled isopropanol was added and incubated at 4°C for overnight.
- The samples were centrifuged for 10 min at 3000 rpm and the DNA pellet was collected and washed with 70% ethanol and air dried.
- The pellet was resuspended approximately in 5 ml of TE buffer depending upon the size of pellet and allowed to dissolve compeletely.
- 15 µl of RNase A (10 mg/ml) was added to remove the RNA and incubated at 37°C for 45 min.
- 500 µl (1/10 volume) of 3M sodium and 10 ml of chilled absolute ethanol was added and allowed to precipitate at room temp for overnight.
- The DNA was hooked out and transferred in 1.5 ml eppendorf tube, washed with 70% ethanol ait dried.
- DNA suspended in 200-500 µl of TE buffer depending upon the pellet size and stored at -20°C.
- Resolve DNA on a 0.8 % agarose gel by electrophoresis when used.

EXPERIMENT - 16

AIM : Agrobacterium tumefaciens-mediated plant transformation. (Indirect gene transfer)

PRINCIPLE : The pathogenic bacteria Agrobacterium have the capacity to transfer part of its plasmid DNA (called the T-DNA) into the nuclear genome of plants cells. Two types of Agrobacterium strains are used for plant genetic transformation. In the A. tumefaciens strains, the T-DNA genes encode oncogenes that will induce the formation of a tumor on the infected plant tissue. In the A. rhizogenes strains, the T-DNA genes encode oncogenes that will induce the production of adventitious roots called the hairy root tissue. This later is used to produce rapidly chimaeric plants with untransformed aerial part and transgenic roots cotransformed with the Ri T-DNA and the construct of interest.

The T-DNA transfer to the plant nucleus depends on the expression of the Agrobacterium vir genes that delimit the extent of the DNA sequence transferred to the nucleus, by recognizing specific sequences called T-DNA right and left borders (RB and LB). In between these borders any DNA sequence can be introduced and transferred into the plant genome. This forms the basis for the generation of transgenic plants.

For this, the oncogenes are deleted from the T-DNA and replaced by selectable marker gene and gene of interest. This T-DNA construct can be placed on another replicon (binary vector) than the vir genes, making the transformation system more versatile. The integration of the T-DNA in the genome probably depends on the plant DNA reparation machinery. Generally one copy of the T-DNA is inserted randomly in the plant genome, and gene fusions studies indicated that these insertions preferably occur in transcribed regions or in their vicinity.

The steps involved are:

1. Infection of plant tissues with overnight grown Agrobacterium culture.
2. Cocultivation.
3. Post-cocultivation wash and Transient expression assay.
4. Culture in selective medium.
5. Selection of putative transformed plants.
6. Molecular analysis of putative transformed plants.

MATERIALS : In vitro germinated seedlings, A. tumefaciens culture, Liquid plant growth medium, Sterilized petridishes, Filter discs, Microtips, GUS substrate,Double distilled water.

WORKING PROTOCOL :

1. Raise the desired Agrobacterium strain in 20 ml of LB medium with appropriate antibiotics, agitated overnight at 200 rpm at 28^0C.
2. Concentrate the cells at 5000 rpm for 5 min, resuspend the cells in liquid plant growth medium.
3. Prepare the explants. Submerge the explants in bacterial suspension for 10-20 min.
4. Blot-dry the explants and cocultivate them in tissue culture growth conditions for 2-3 days.
5. Wash the explants with sterile dd water to eliminate Agrobacteria.
6. Incubate few explants in GUS substrate (overnight in the dark at 37^0C) after for detection of transient GUS expression.

RESULT : Strong expression of GUS (indigo blue color) was observed in the region of the explants from where the shoots developed. The endogenous GUS activity (color) was not detected in non-transformed (control) explants. GUS activity at the cut ends indicates the susceptibility of explants to Agrobacterium mediated transformation.

EXPERIMENT - 17

AIM : Direct DNA delivery to plant by Particle Bombardment

PRINCIPLE : The fact that DNA could be delivered into plant cells by physical means and expressed in intact cells effectively, revolutionized genetic engineering of plants. Out of the available physical procedures for delivering DNA, particle bombardment is the most preferred method as it allows introduction of DNA directly into any plant cell type. With particle bombardment, the difficulties of using fragile protoplasts and host-range limitations associated with Agrobacterium are circumvented.

The basis of the particle bombardment process is the acceleration of DNA-coated microprojectiles (mainly particles of tungsten or gold, with 0.2 to 1.5 μm in diameter) at high speed (about 1500 km/h) towards the living cells. After penetration in the cell, the DNA dissociates from the microprojectiles and integrates into the chromosome.

The BioRad PDS1000/He biolistic gun will be used for the said purpose, which is a gas (He) pressure-driven device. It uses plastic film as a macro carrier. The device is powered by a burst of helium gas that accelerates the supporting macrocarrier onto which DNA coated microcarries are loaded. The pressure at which helium bursts is controlled by rupture disk (made up of kapton membrane). The rupture disk can be choosen such that helium could be allowed to burst at different pressures. Upon bursting of helium gas, the macrocarrier is instantly accelerated but is stopped by the stopping screen (metal perforated screen). The microcarriers then pass through the stopping screen due to their small size and move at desired velocity. The air in the chamber is evacuated by vacuum suction to reduce the airdrag, which might slow down the velocity of the microcarriers.

Biolistic Gun

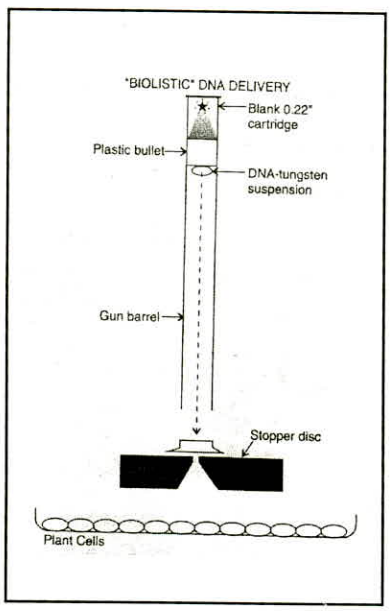


Fig.17.1. The gene gun

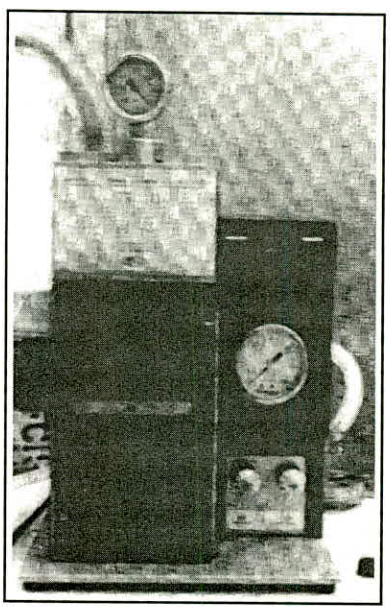


Fig. 17.2. PDS 1000 Particle gun

MATERIALS : Explants, Microcarrier (gold particles), Plasmid DNA with reporter gene, Macrocarrier, Stopping screen, Macrocarrier launch assembly, Biolistic Gun

INSTRUCTION :

- Soak macrocarriers, holders, stopping screens, rupture disks in 95% ethanol for 15 min, then air dry.
- Coat plasmids over gold particle and prepare a suspension.
- Drip 6 ~ 10 µl of the suspension on to the macrocarrier.
- Open the valve on the steel cylinder, which contain the pressurized helium, rotate the black button (helium pressure regulator) to adjust the helium pressure (at least 200 psi higher than the desired pressure).
- After all the materials are in place, close the chamber door and apply vacuum.
- When appropriate vacuum is reached, activate the fire switch. The gas is held until the burst pressure of the rupture disk is reached.

RESULT : Strong expression of GUS (indigo blue color) was observed in the bombarded cells of the explants. The endogenous GUS activity (color) was not detected in non-transformed (control) explants i.e, explants bombarded with naked particles. GUS activity in the bombarded cells indicates the direct gene delivery to the target plant cells.

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1. H.S. Chawla,2005. Laboratory manual for Plant Biotechnology. Oxford and IBH Co. Pvt. Ltd., New Delhi.
2. T. Pullaiah and M.V. Subba Rao, 2009. Plant Tissue culture Theory and Practices, Scientific Publishers (India), Jodhpur.
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4. Akshya Talukdar, 2011. Practical handbook on Molecular Marker Techniques for Crop Improvement.
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EXPERIMENT - 18

Confirmation of Genetic transformations

AIM : Molecular analysis of putative transformed plants by Polymerase Chain Reaction.

PRINCIPLE : Detection of transgenes, which may not be being expressed at that time, can only be achieved by analysis of plant DNA. By their very nature, transgenes are novel, and can be distinguished from the surrounding host plant genome, but at the practical level this requires either some knowledge of the inserted DNA sequences.

The most common strategy employed for screening of transgene presence is PCR-based detection of transgenes followed by gel electrophoresis and comparison with standard samples. The process uses the enzyme Taq DNA Polymerase to amplify minute quantities of transgene DNA from plant material to a detectable level. A major advantage of a PCR-based detection-strategy is that it is extremely sensitive.

PCR is usually conducted in microtubes or microtitre plates, and reaction volumes vary from 10 to 100µl. The quantity of template DNA used also varies considerably. PCR reaction schemes differ with respect to times, temperatures, and numbers of amplification cycles, often for the same assay in different laboratories. Most PCR tests are assessed by agarose gel electrophoresis, and results are scored visually as the presence or absence of a DNA fragment of the appropriate size.

The quantity of template plant DNA used in the PCR ranges from 5 - 100ng. Primer concentrations used vary from 2 - 10.0 µM. In practice, primer lengths are normally around 20 - 25 nucleotides (the shortest reported being 16). PCR reaction schemes are broadly similar, reaction times varying with the thermocycler used, and ramping rates being set as fast as possible. Primer annealing temperatures for most assays are standardised at approximately 55°. Most assays use 30-35 amplification cycles, although some labs use particular assays of 45-50 cycles. This may increase the sensitivity of the test, but care is necessary in these extended runs as the effect of minor contamination or PCR artefacts is significantly amplified. PCR results are assessed by gel electrophoresis (1.4 - 4.0% agarose).

A positive PCR result only means that a product has been successfully amplified, but the host plant DNA template may not necessarily be the source. Likewise, a negative PCR result only indicates that a product has not been amplified. It does not necessarily imply that the transgene is not present. These problems are addressed by the use of duplicate samples and appropriate controls. Each PCR run performed includes the following controls :

- Verified positive control.
- Verified negative leaf sample.
- No-DNA blank controls.

MATERIALS :

- Genomic DNA isolated from control plant (untransformed).
- Genomic DNA isolated from putative transformed plants.

- PCR components.
- Thermal Cycler

INSTRUCTION :

1. To perform several parallel reactions, prepare a master mix containing water, buffer, $MgCl_2$, dNTPs, primers and Taq DNA Polymerase in a single tube, which can then be aliquoted into individual tubes.
2. Add the desired amount of master mix to the template DNA. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.
3. Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube.
4. Set the conditions in Thermal cycler, place the samples and start PCR.

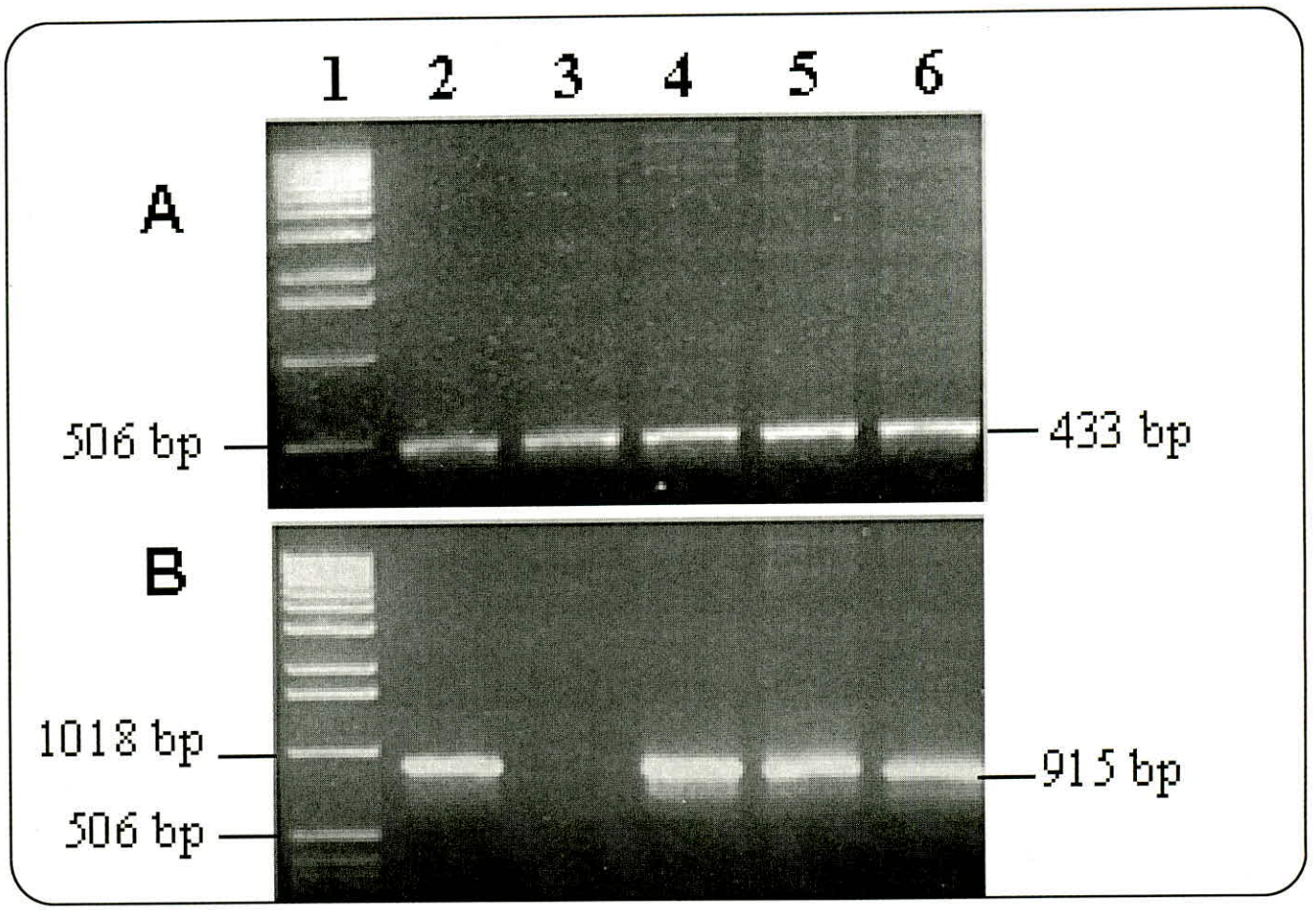


Fig.: PCR screening of some transgenic wheat plants using different PCR primers.

Lane 1:1Kb DNA Ladder; lane 2: positive control (plasmid); lane 3: negative control (nontransgenicplant); lanes 4-6: transgenic wheat plants; A: PCR amplifications using DyFand DyR primers; B: PCR amplifications using KS and Dy10R1 primers.

EXPERIMENT - 19

AIM : Demonstration of Gel Electrophoresis Techniques.

PRINCIPLE : Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. We will be using agarose gel electrophoresis to determine the presence and size of PCR products.

Background : Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Purpose : To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

MATERIALS : Agarose, TBE buffer, Gel casting tray, comb, power pack (Gel electrophoresis unit), Sample DNA, Molecular Weight marker, Loading dye, Sterile microtips, EtBr staining solution, UV transilluminator or Gel Documentation System.

Agarose Gel Electrophoresis Protocol

Preparing the agarose gel

- Measure 1.25 g Agarose powder and add it to a 500 ml flask.
- Add 125 ml TAE Buffer to the flask. (the total gel volume well vary depending on the size of the casting tray).
- Melt the agarose in a microwave or hot water bath until the solution becomes clear. (if using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).
- Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
- Seal the ends of the casting tray with two layers of tape.
- Place the combs in the gel casting tray.
- Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
- Carefully pull out the combs and remove the tape.
- Place the gel in the electrophoresis chamber.
- Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note : gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

- Add 6 l of 6X Sample Loading Buffer to each 25 l PCR reaction.
- Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.
- Carefully pipette 20 l of each sample/Sample Loading Buffer mixture into separate wells in the gel.
- Pipette 10 l of the DNA ladder standard into at least one well of each row on the gel.

Note : if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel.

Running the gel

- Place the lid on the gel box, connecting the electrodes.
- Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected. (Remember – "Run to Red").
- Turn on the power supply to about 100 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – **it should not exceed 5 volts/ cm between electrodes!**.
- Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.
- Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
- Let the power run until the blue dye approaches the end of the gel.
- Turn off the power.
- Disconnect the wires from the power supply.
- Remove the lid of the electrophoresis chamber.
- Using gloves, carefully remove the tray and gel.

Gel Staining

- Using gloves, remove the gel from the casting tray and place into the staining dish.
- Add warmed (50-55°) staining mix.
- Allow gel to stain for at least 25-30 minutes (the entire gel will become dark blue).
- Pour off the stain (the stain can be saved for future use).
- Rinse the gel and staining tray with water to remove residual stain.
- Fill the tray with warm tap water (50-55°). Change the water several times as it turns blue. Gradually the gel will become lighter, leaving only dark blue DNA bands. Destain completely overnight for best results.
- View the gel against a white light box or bright surface.
- Record the data while the gel is fresh, very light bands may be difficult to see with time.

Note : Gels stained with blue stains are stable for long periods. When destaining is complete, remove gel from water and allow the gel to dehydrate. Dark bands can be seen for in a dried gel for weeks or months.

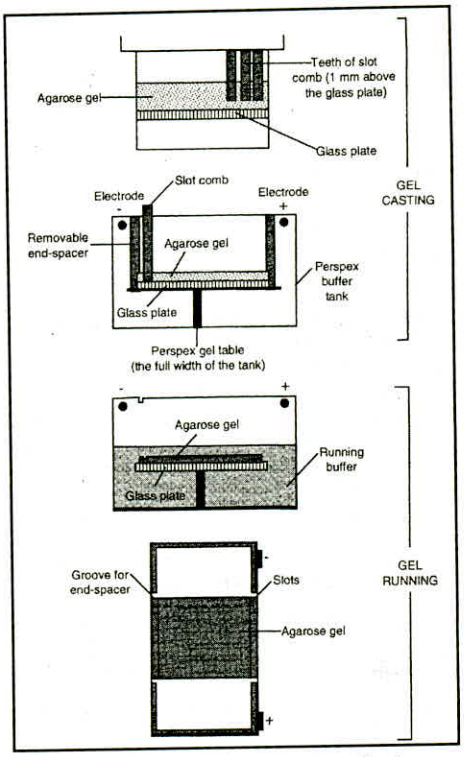
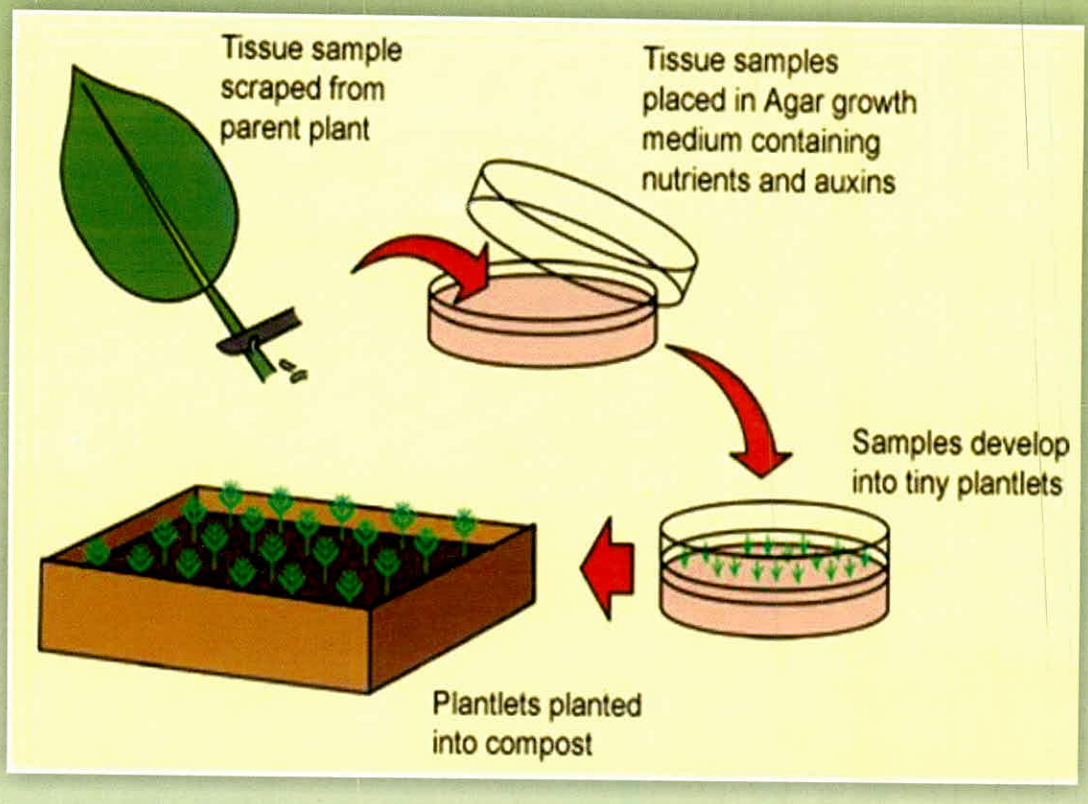
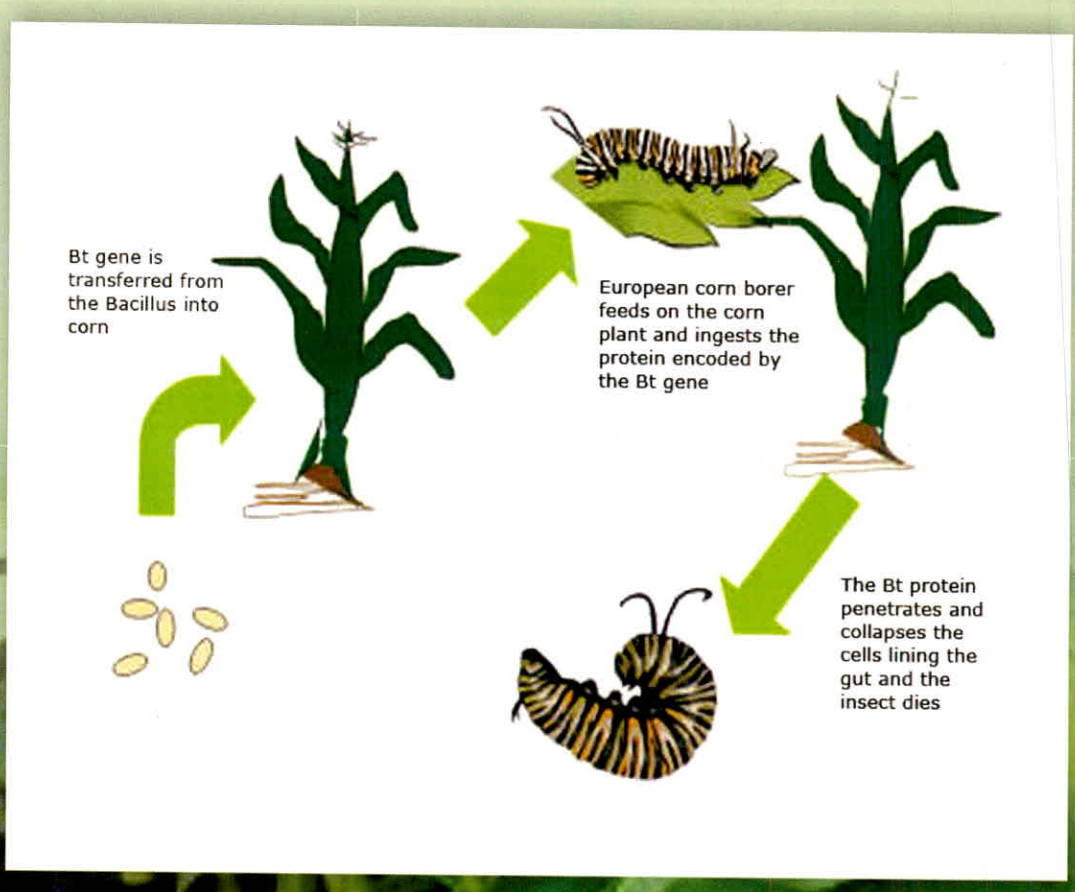
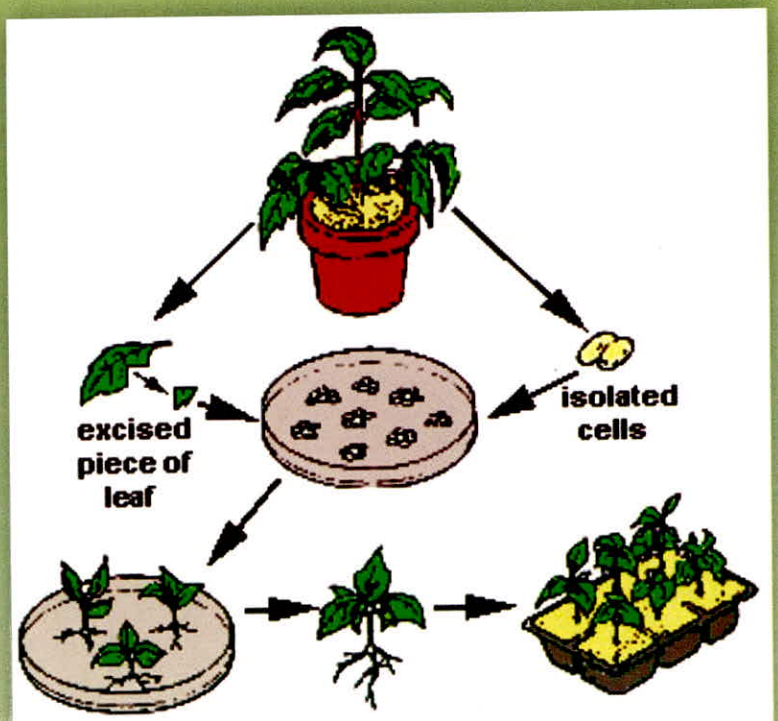


Fig. 19.1. Agarose Gel Electrophoresis

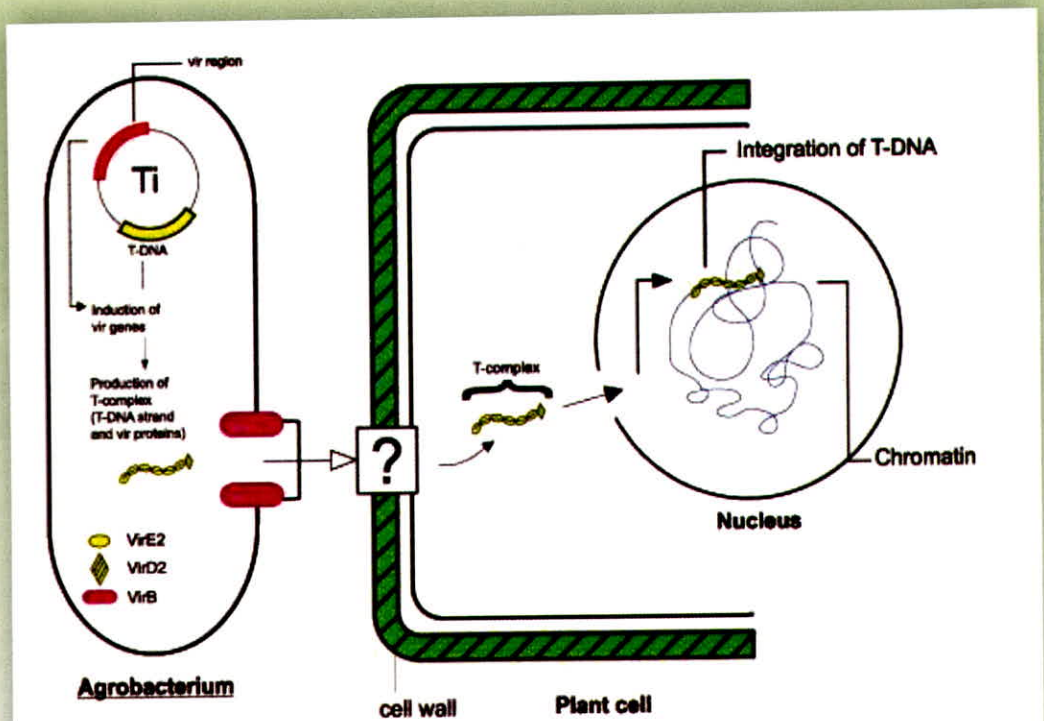




Effect of Bt gene in Transgenic Plants



Overview of the Tissue Culture Process



T-DNA transfer into the Plant's Genome

Adapted from Zupen et al 2000