Plant cellular breeding

APPLICATION OF CELLULAR MANIPULATIONS

In-vitro selection

one of somaclonal variation method. Its effectiveness and efficiency are due to its ability of changing the plant to the desired character, either by applying a selection agent on the culture media or by giving particular condition to change the somaclone with the required character

BREEDING METHODS

1 Direct (positive) selection, in which survive of a certain type of mutant cells;

2 Indirect (negative) selection, based on a selective destruction of dividing cells of wild-type and survival of metabolically inactive cells, but require additional identification of their mutational changes;

3 Total selection, in which all individually cell clones are tested;

4 Vizual selection and non-selective selection when the mutant line can be identified among

The most common is the direct selection based on the selection of cells with desired traits for resistance to stress factors (pathogens, herbicides, salinity, non-optimal temperature, high or low pH, heavy metals, etc.). Typically, selective agents are added to the nutrient medium. To highlight somaclones resistant to pathogens, use the medium to a toxin secreted by the pathogen or a culture broth (medium, filtrate) in which developing pathogen. To create forms that are resistant to salinity, in the culture medium was added salt NaCl, $Na_2 SO_4$.

For selection of forms with a high content of essential amino acids added to the medium of the toxic counterparts (analogs). At this time there is an accumulation of a basic amino acid in the cell. For example, resistant carrot cells to metioninsulfoksil comprise in 200 times more methionine, and aminoethylcysteine-resistant cells are accumulated lysine in a 50-fold higher than in controls (Shamina, 1984).

Наиболее распространенной является прямая селекция, основанная на отборе клеток с желаемыми признаками на устойчивость к стрессовым воздействиям (патогены, гербициды, засоление, неоптимальная температура, высокие или низкие значения рН, тяжелые металлы и др.). Как правило , селективные агенты добавляют в состав питательной среды . Для выделения сомаклонов, устойчивых к патогенам , используют среду с токсином, выделяемым патогеном или с культуральной жидкостью, в которой развивался патоген. Для создания форм, устойчивых к засолению, в состав питательной среды добавляют соли Na Cl, Na_2SO_4.

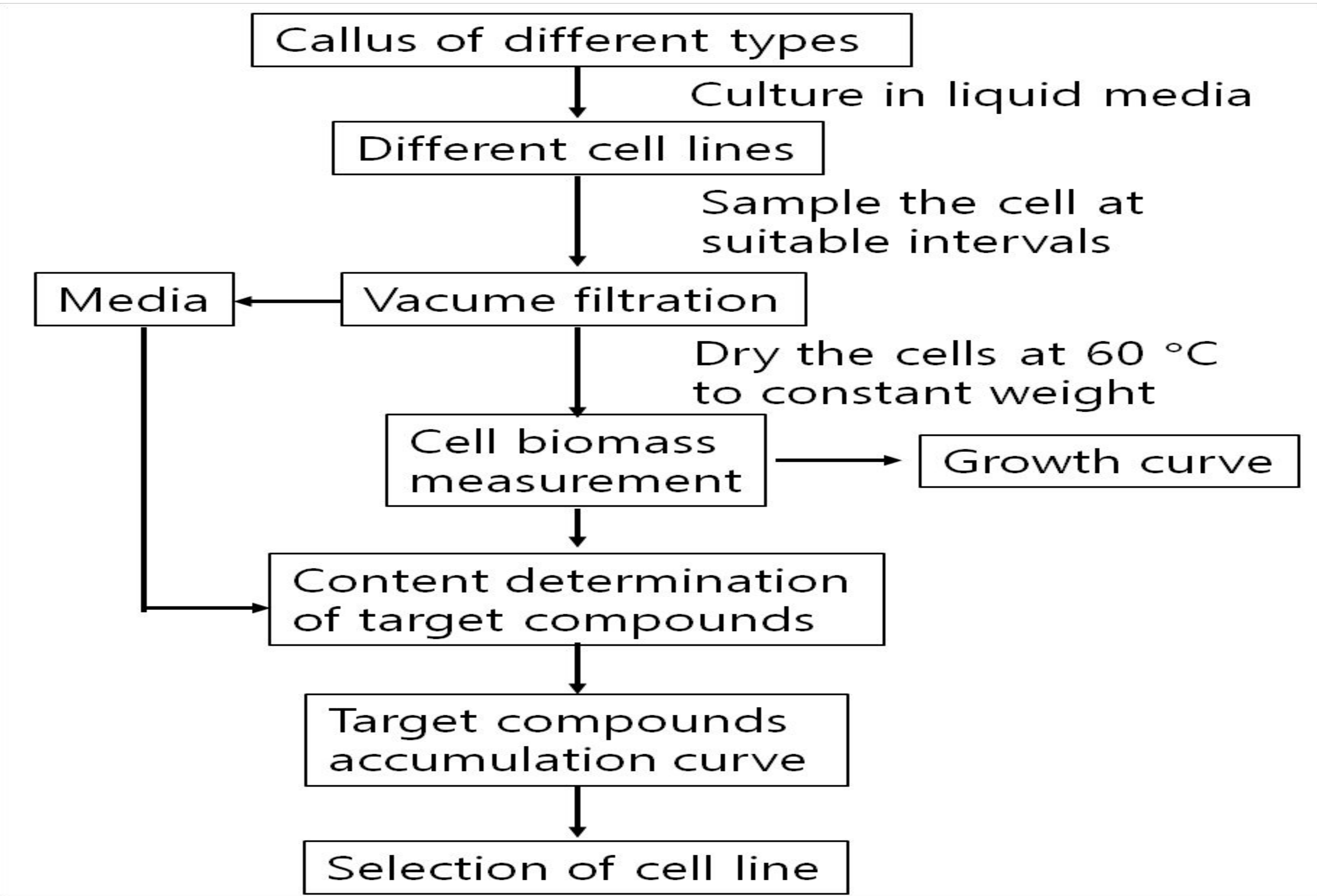
Для отбора форм с повышенным содержанием незаменимых аминокислот в среду добавляют их токсические аналоги. При этом происходит накопление в клетке основной аминокислоты. Так, например, резистентные к метионинсульфоксилу клетки моркови содержат в 200 раз больше метионина, а резистентные к аминоэтилцистеину накапливают лизина в 50 раз больше, чем в контроле (Шамина, 1984).

Scheme of direct selection of mutants is as follows? (Sidorov, 1990):

1. Allocation of callus protoplasts or suspension culture;
2. Occurrence of spontaneous somaclonal variability in cell culture, or inducing chemical or physical mutagens,
3. Culturing cells in non-selective conditions;
4. Culturing cells on selective media (selection of cell variants);
5. Culturing of the colonies of cells on the regeneration medium;
6. Regeneration of plants and their propagation (tests for stability, biochemical analyzes);
7.Vysadka plants in soil (testing to resistance of seeds, the study of the genetic nature of resistance).

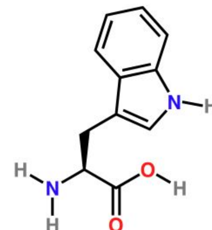
Схему прямой селекции мутантов можно представить следующим образом (Сидоров, 1990):

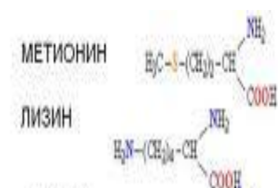
1.Выделение каллуса, протопластов или суспензионной культуры;
2. Возникновение спонтанной сомаклональной изменчивости в культуре клеток, или ее индуцирование химическими или физическими мутагенами;
3. Культивирование клеток в неселективных условиях;
4. Культивирование клеток на селективных средах (отбор клеточных вариантов);
5. Культивирование колоний клеток на регенерационных средах;
6. Регенерация растений и их размножение (проведение тестов на устойчивость, биохимические анализы);
7.Высадка растений в почву (тестирование на устойчивость семян, изучение генетической природы устойчивости).


Callus of different types
Culture in liquid media
Different cell lines
Sample the cell at
suitable intervals
Media
Vacume filtration
Dry the cells at 60 °C
to constant weight
Cell biomass
measurement
Growth curve
Content determination
of target compounds
Target compounds
accumulation curve
Selection of cell line

Obtained by the method of cell selection: maize line resistant to helminthosporiosis; line potatoes: resistant to late blight; tobacco plants resistant to tobacco mosaic virus. In cell culture derived mutants with increased synthesis of essential amino acids. Thus, the selected carrot cells and tobacco, are synthesized in 20-30 times more free tryptophan in comparison to the original parent cultures. A number of cell lines of potatoes, carrots, rice, capable supersynthesis lysine, methionine, proline, phenylalanine, glycine was produced by this method.

Методом клеточной селекции получены: линии кукурузы, устойчивые к гельминтоспориозу; линии картофеля, резистентные к фитофторе; растения табака, устойчивые к вирусу табачной мозаики. В культуре клеток получены мутанты с повышенным синтезом незаменимых аминокислот. Так, отобраны клеток моркови и табака, синтезирующие в 20-30 раз больше свободного триптофана по сравнению с исходными родительскими культурами. Этим способом получен целый ряд клеточных линий картофеля, моркови, риса, способных к сверхсинтезу лизина, метионина, пролина, фенилаланина, глицина



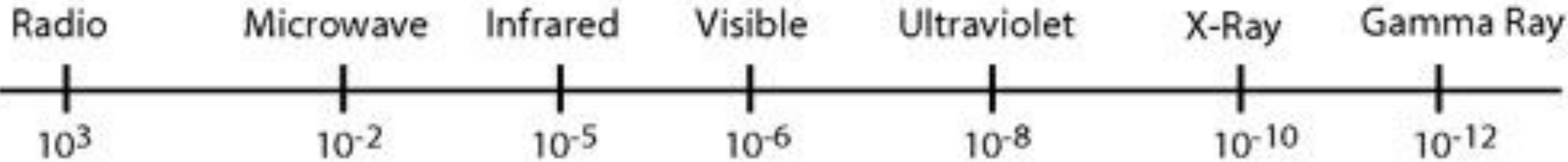


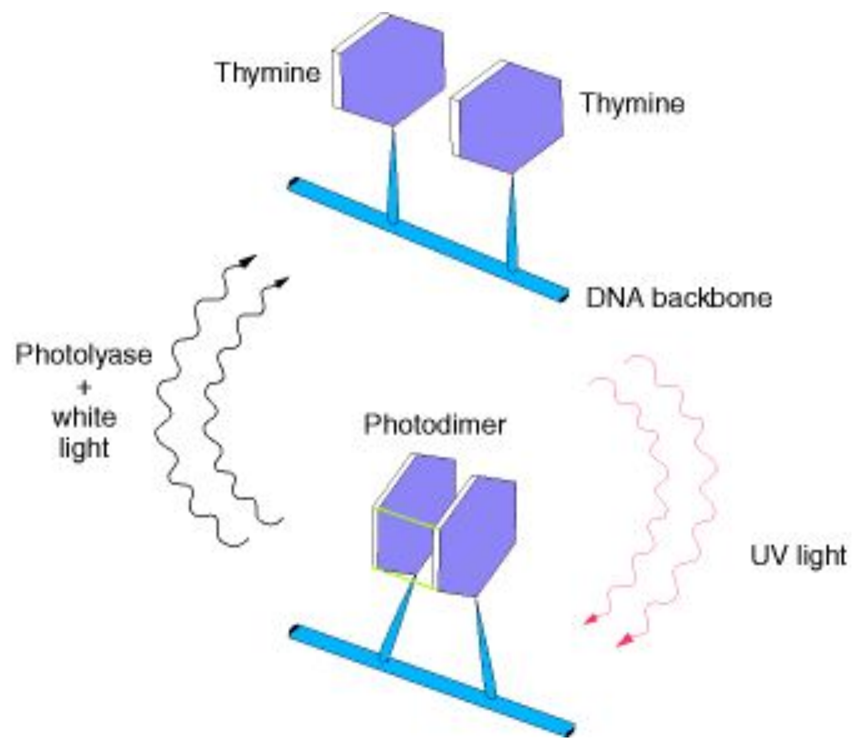
In-Vitro mutant selection for biotic stresses in Plants

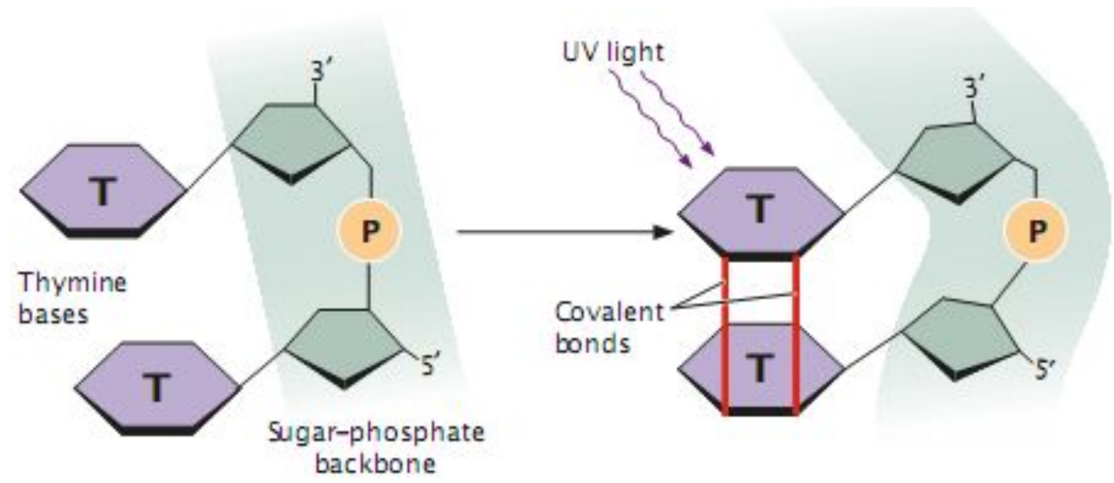
Type of Mutation Induction

1. Physical Agents
2. Chemical Mutagens
3. Colchicine
4. Transposan mediated Mutagenesis
5. Site Directed Mutagenesis
6. Somaclonal variation

Physical Agents




Thymine
Thymine
DNA backbone
Photolyase
+
white
light
Photodimer
UV light


UV light
3′
3′
T
T
T
T
P
P
Thymine
bases
Covalent
bonds
5′
5′
Sugar–phosphate
backbone

Gamma Irradiation Chambers

Chemical Mutagens

1. Base analogs

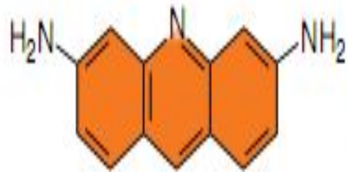
bromouracil (BU) **aminopurine** (AP)

2. Chemicals which alter structure and pairing properties of bases

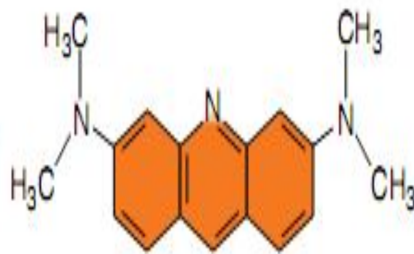
Alkylating agents [ethyl methanesulphonate (**EMS**); diethyl sulphate (dES); ethyleneimine (EI); ethyl nitroso urethane (ENU), ethyl nitroso urea (ENH), methyl nitroso urea (MNH)

3. Intercalating agents - molecules capable to embed (intercalate) between two comple-mentary base pairs in double-stranded DNA or RNA. **Acridine orange, Proflavin, Ethidium Bromide**

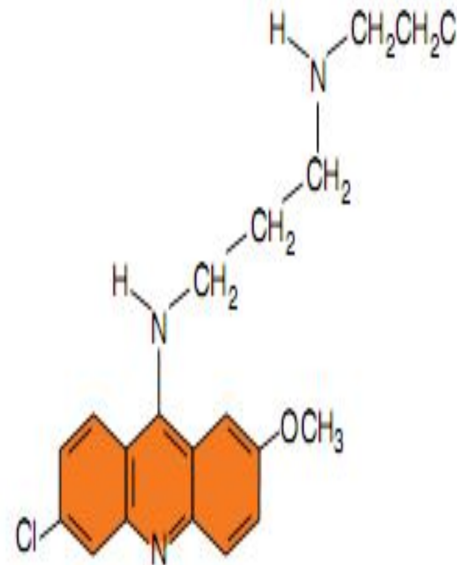
Intercalating Agents



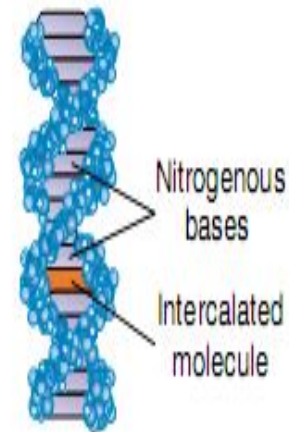
Proflavin

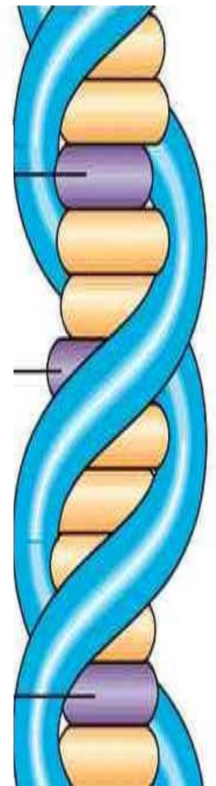


Acridine orange



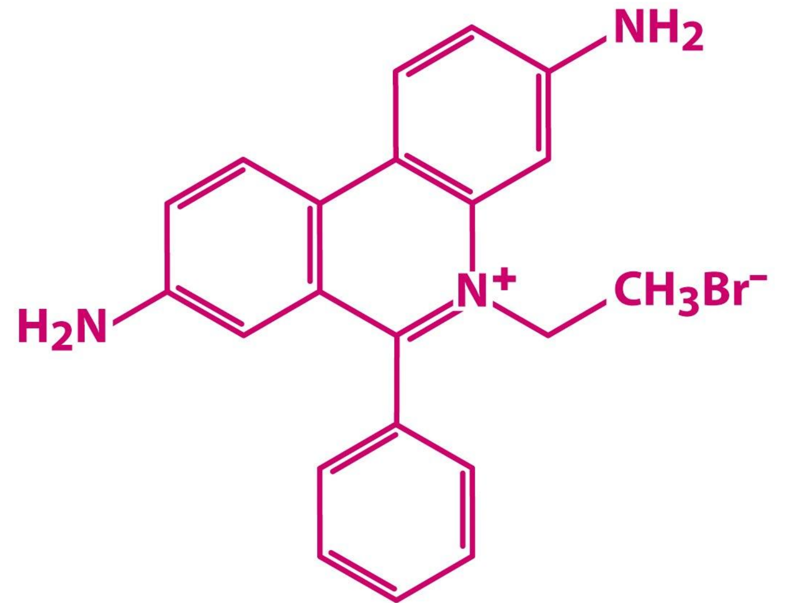
ICR-191





Ethidium bromide

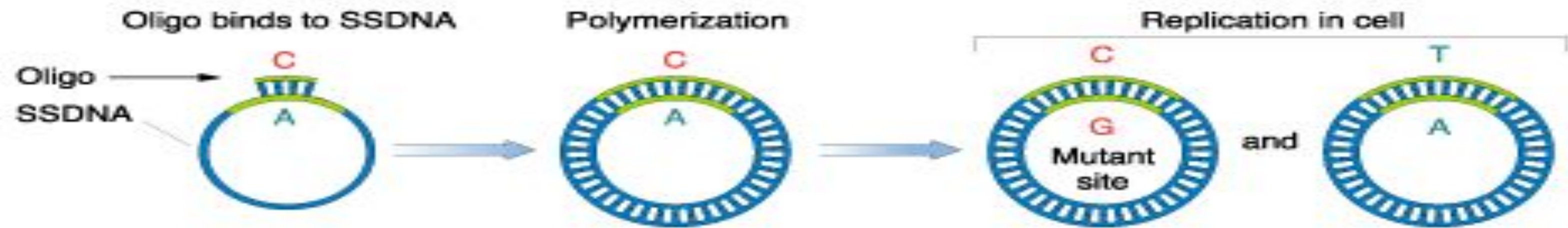
Ethidium bromide



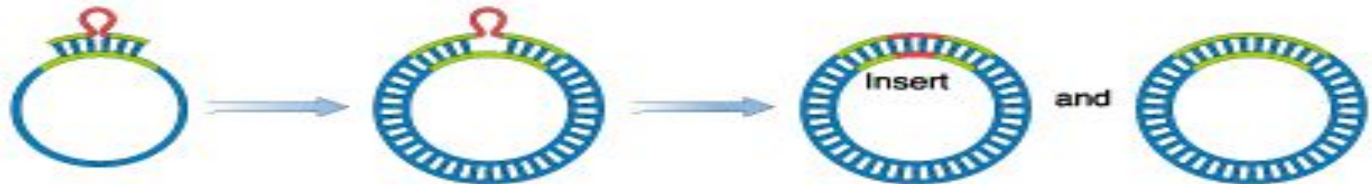
Site Directed Mutagenesis

(a) Oligonucleotide-directed mutagenesis

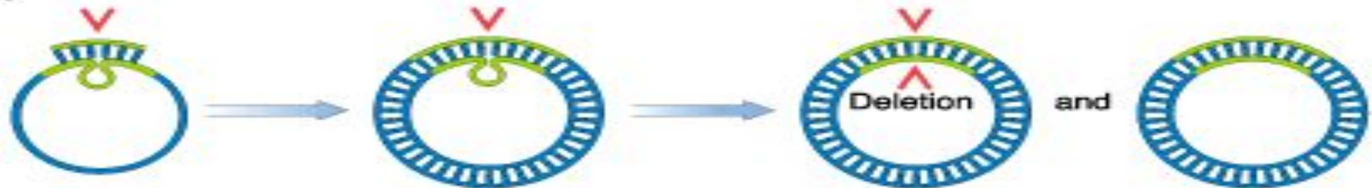
(i) Base-pair substitution



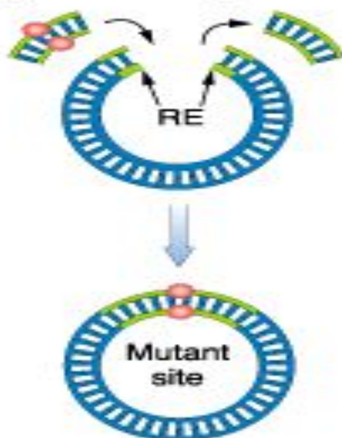
(ii) Insertion



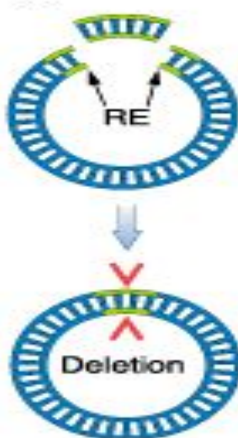
(iii) Deletion



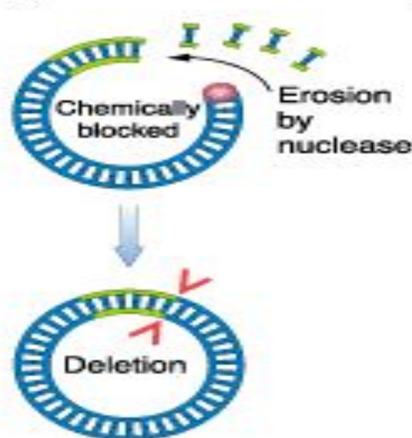
(b) Cassette replacement



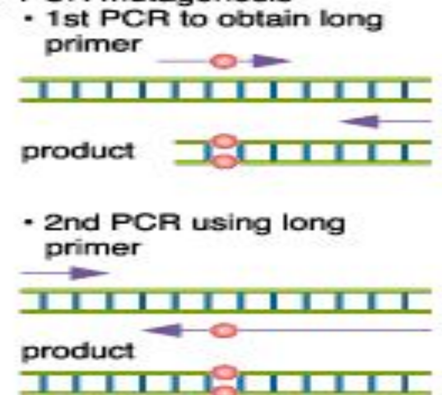
(c) Deletion

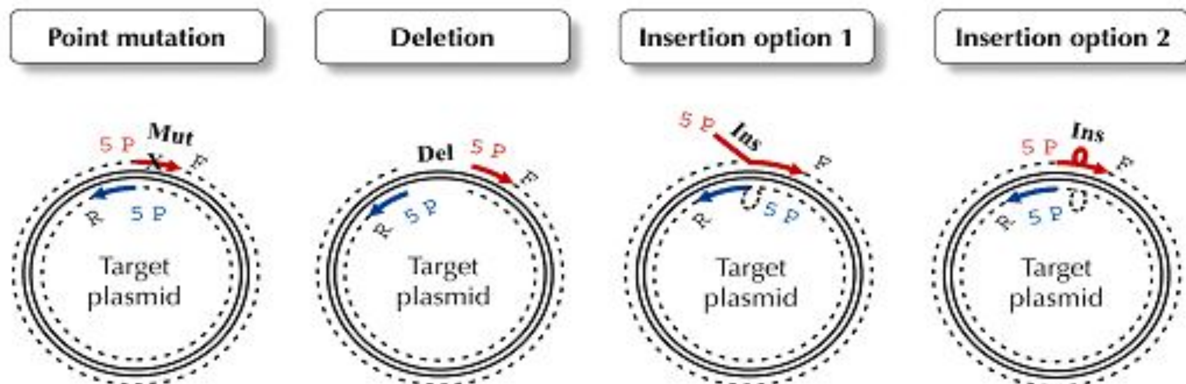


(d) Sets of deletions



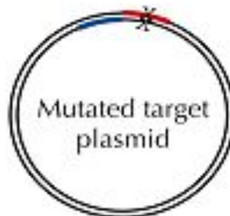
(e) PCR mutagenesis



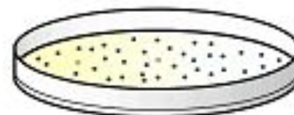


Linear amplified target plasmid
with desired mutation

Step 1.
Amplification of target plasmid with two phosphorylated primers.

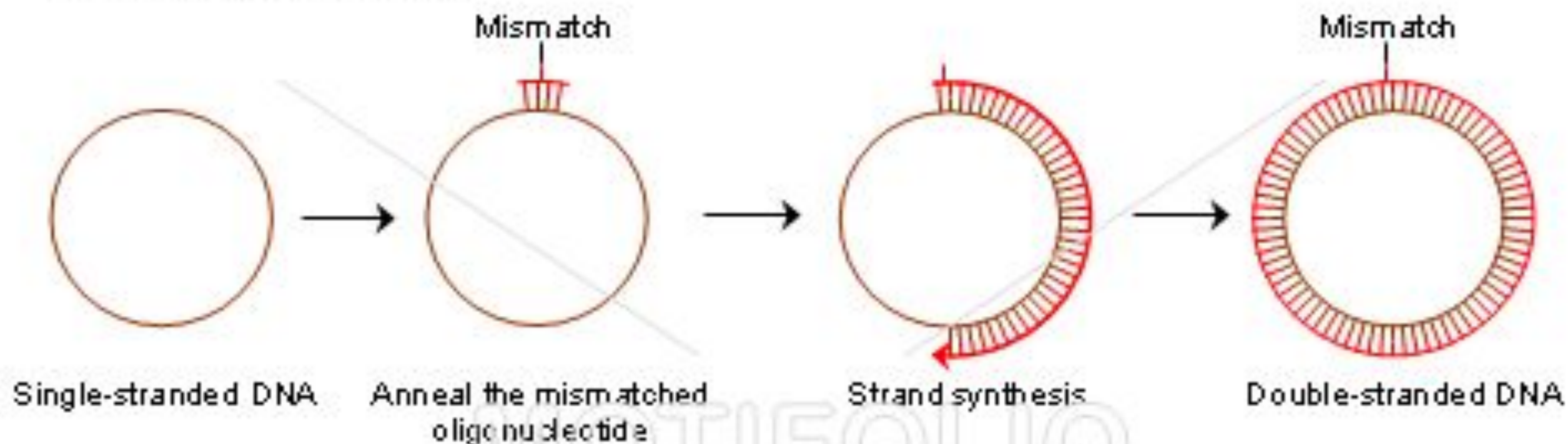


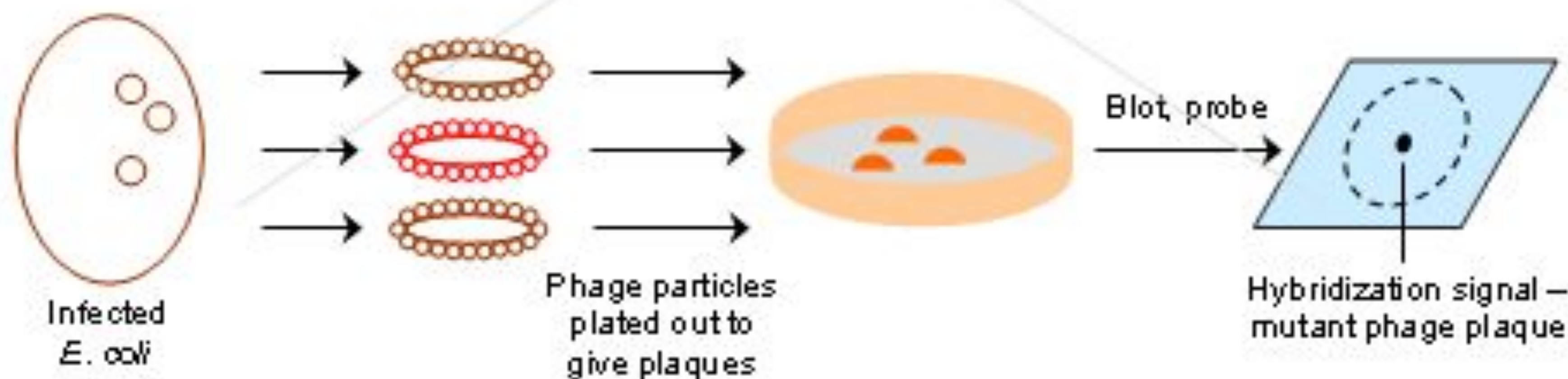
Step 2.
Plasmid circularization by ligation.



Step 3.
Transformation into *E. coli*.

Site-directed mutagenesis

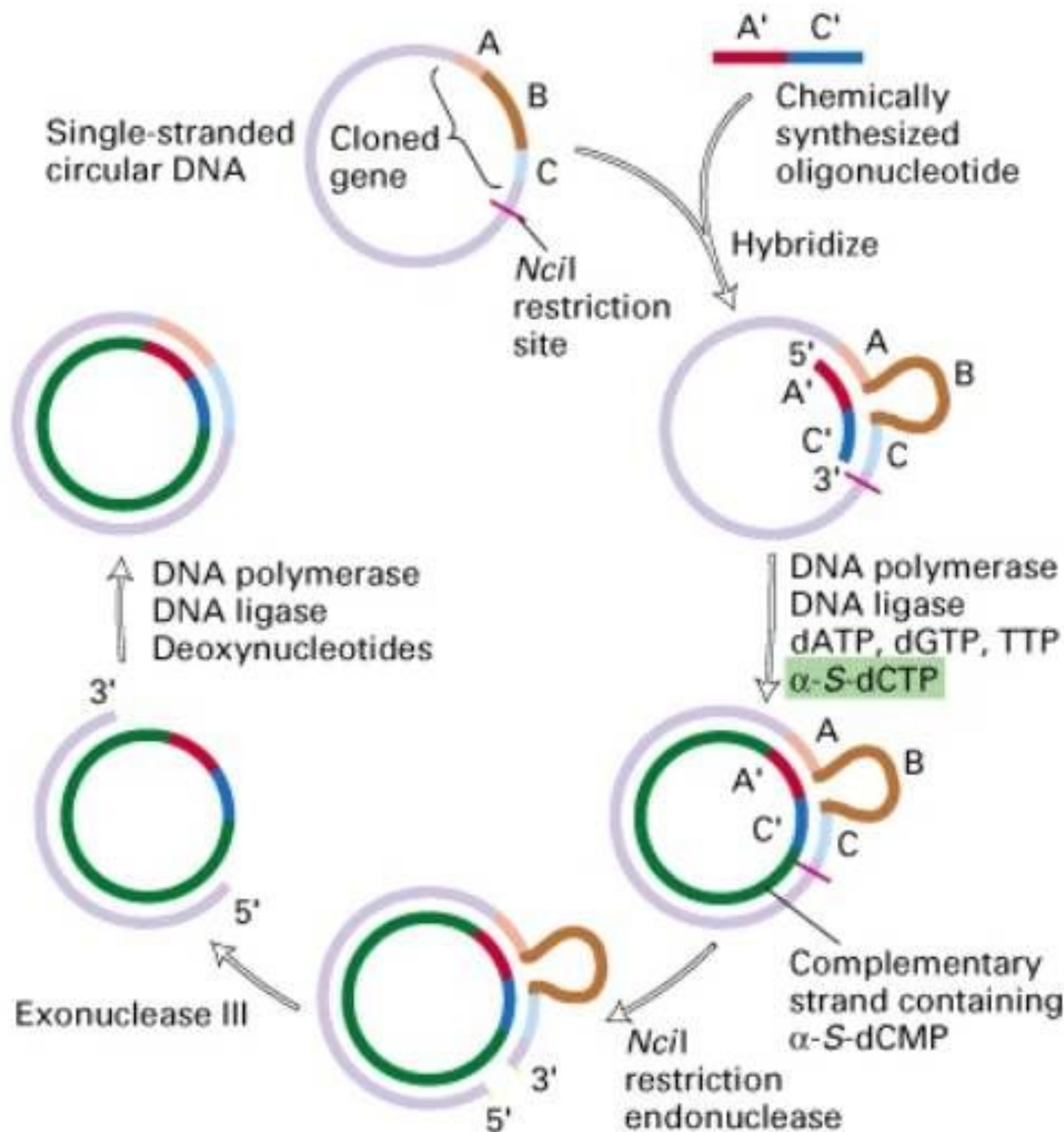




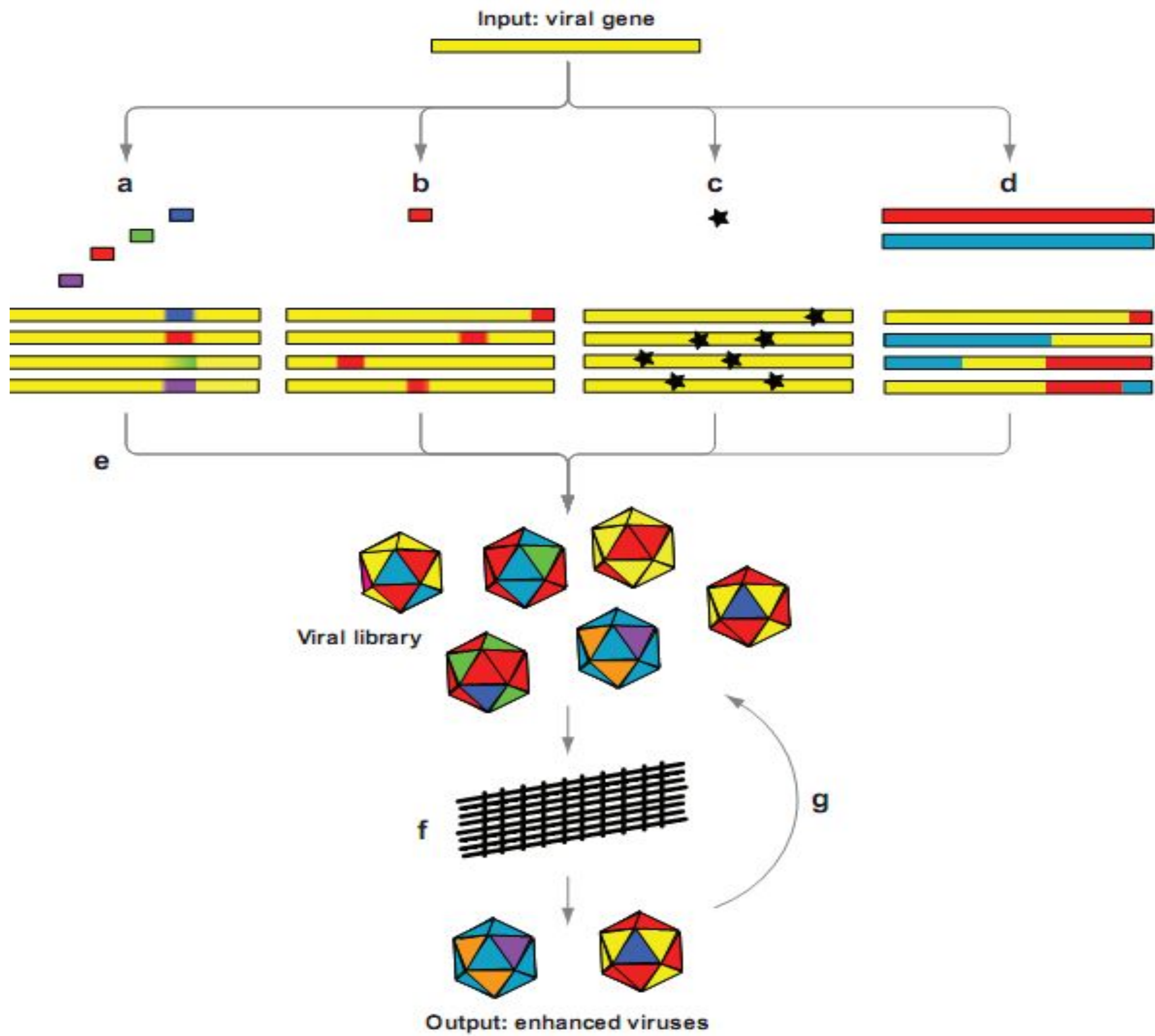
6111153

Copyright © motifolio.com

Oligonucleotide-based mutagenesis is the most commonly used method to introduce mutations in coding sequence.



Enhanced Genome



Site- Directed Mutagenesis (SDM)

sometimes called *site-specific mutagenesis*, is a process that produces mutations in DNA that are controlled by us.

Protein Engineering - one of the most sophisticated applications of recombinant DNA technology - where the properties of a protein, such as an enzyme, are altered in an attempt to 'improve' it by changing (mutating) the gene coding for the protein using SDM.

Desired improvements might be increased thermostability, altered substrate range, reduction in negative feedback inhibition, altered pH range, *etc*.

Choice of Explant for *invitro* mutagenesis

Choice of Plant Material

A preliminary key step toward the successful use of micropropagation for mutation breeding is the choice of the mother plant, since the starting material should provide a reliable **genetic basis** and high phytosanitary levels (Ahloowalia, 1998).

Virus- free mother plants are the best starting material for tissue culture initiation.

Somatic Embryos:

This system, based on single-cell origin of regenerated plants, allows the treatment of large populations and the rapid generation of homo-histonts (i.e., **non-chimeric** plants).

Advantages of somatic embryogenesis

(a) Originate from a single cell, which minimises or **eliminates chimera**, depending on the plant species

(b) Somatic embryo **cell suspension** is ideal for mutation induction due to the production of direct mutant somatic embryos

(c) Behave like zygotic embryos in germination

(d) Single somatic embryo can be **encapsulated**

(e) Most suitable approach for plant regeneration of **woody species**

(f) Somatic embryos can be produced in a **bioreactor**, which can be automated for large scale production of somatic embryos

In-vitro mutagen treatment

Mutagen treatment of in vitro tissues enhances the frequency rate of spontaneous mutations that may result in a range of mutation spectrum (Jain and Maluszynski, 2004).

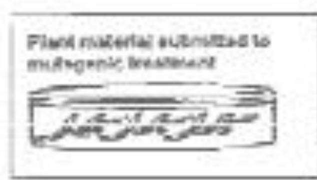
Chemical mutagens

- easy to handle, especially when using cell suspension.
- don't require special equipment used for radiation treatment.

Normally, chimeras are a major problem in regenerated plants by mutagen treatment of multi-cellular structures, such as shoot tips or axillary buds. Mutagen treatment of shoot tips or other organs leads to chimeras that require repeated vegetative propagation up to M_1V_4 level in order to dissociate the chimeras (Jain, 2000; Predrieri, 2001).

Roux et al. (2001) reduced cytochimeras by colchicine treatment in three banana micropropagation systems shoot tip culture, using multi-apexing culture and a corm slice technique.

plant breeders prefer solid or periclinal mutants to developing mutant cultivars.



TISSUE EXPOSED TO MUTAGENS

tissue derived from treated meristem

Subculture interval

Transfer to a fresh medium

vM_{1a} vM_{1b} vM_{1c}

PM PM

vM_{2a} vM_{2b} vM_{2c}

vM_{3a} vM_{3b} vM_{3c}

$vM_{n\,a}$ $vM_{n\,b}$ $vM_{n\,c}$

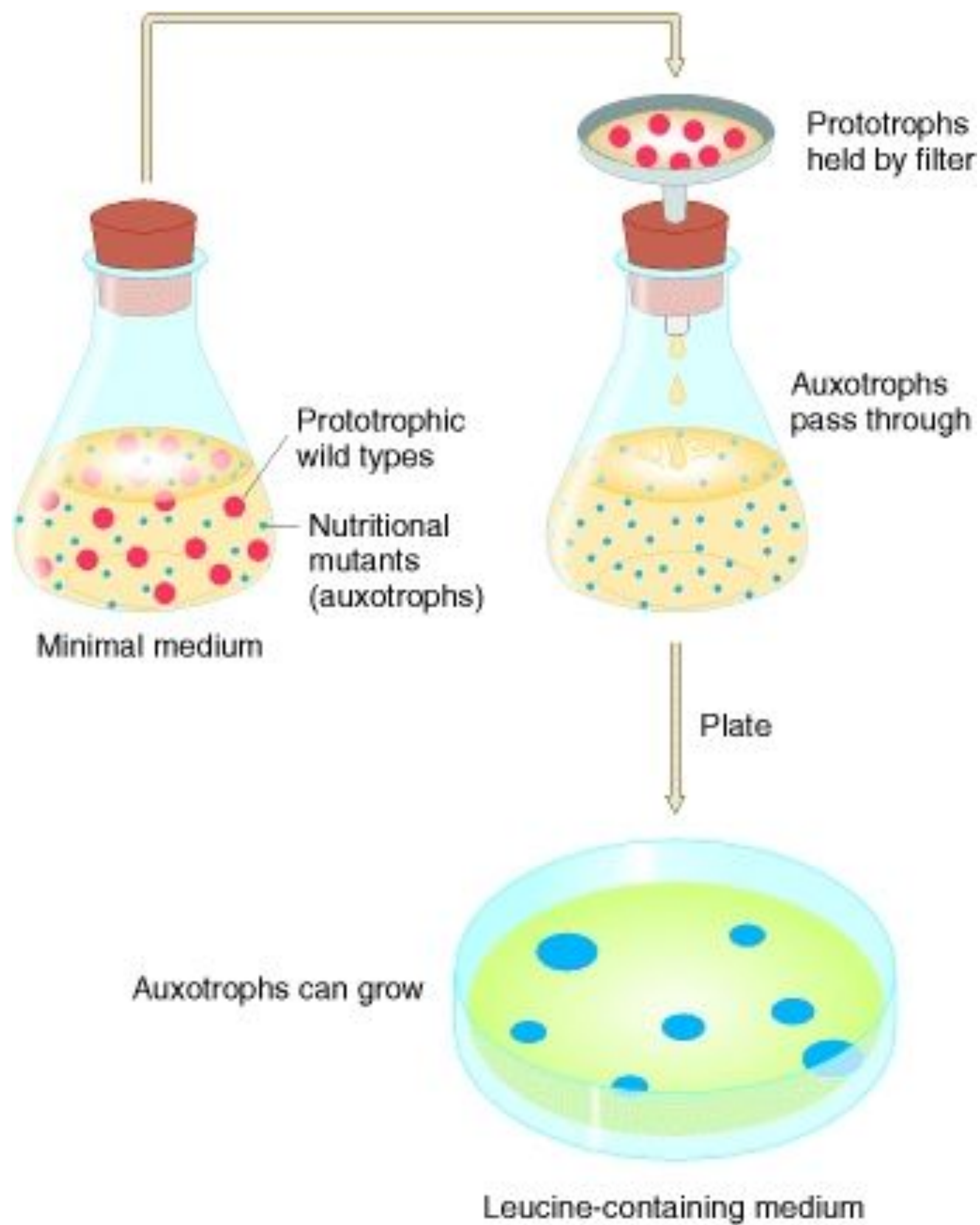
Subculture in presence of a selecting factor

Rooting and subsequent selection in vitro or in vivo

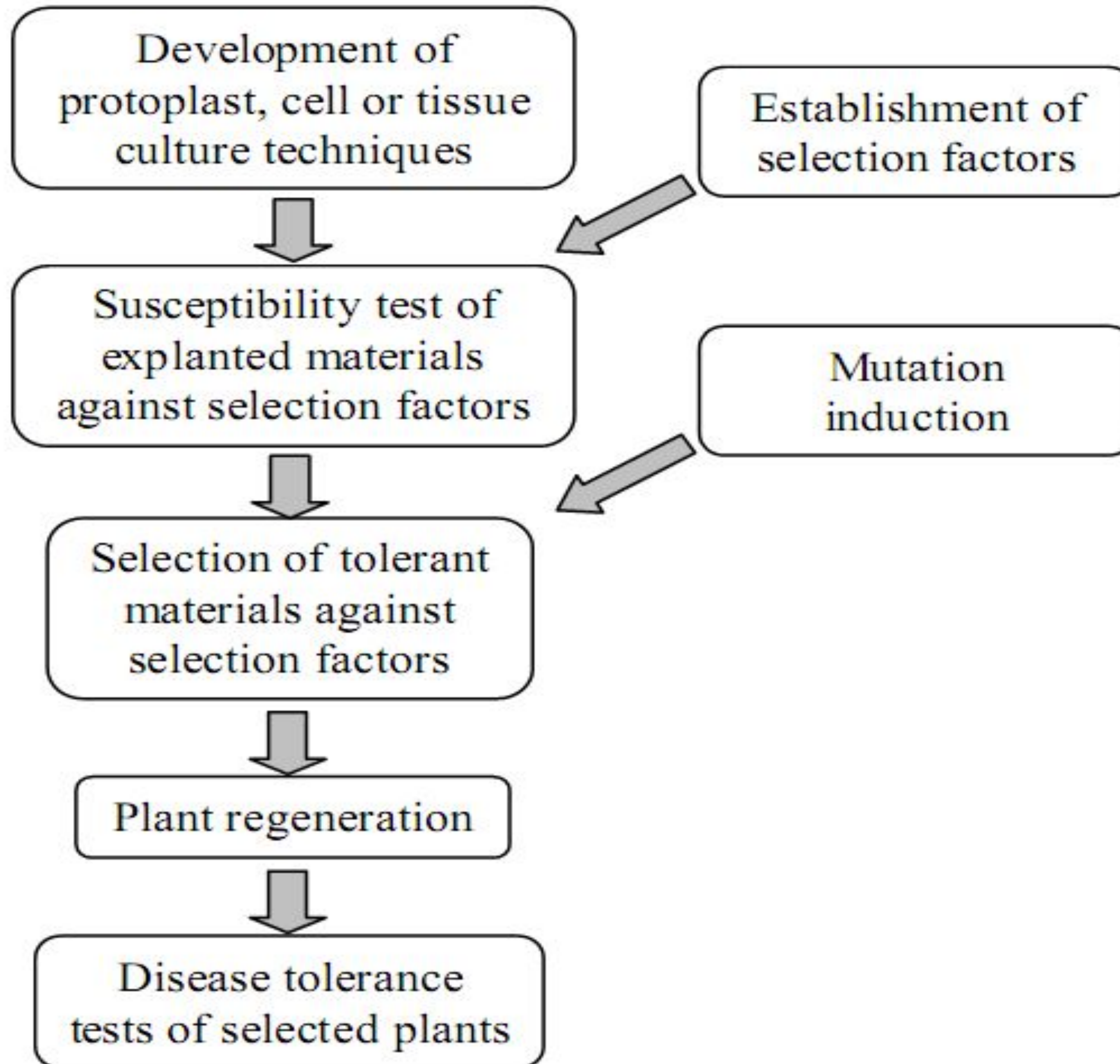
In-vitro selection

It can be employed for selection for both Biotic stress resistance and Abiotic stress tolerance

First *In-vitro* selection for disease resistance conducted by Carlson (1973) for Tabotoxcin (Methionine sulfoximine) produced by *Pseudomonas syringae p. var. tabaci in* Tobacco


Prototrophs
held by filter
Auxotrophs
pass through
Prototrophic
wild types
Nutritional
mutants
(auxotrophs)
Minimal medium
Plate
Auxotrophs can grow
Leucine-containing medium

In vitro selection process to obtain disease resistance plants



General Scheme of in vitro screening for disease resistance and Selection

PLANT

Establishment of explant culture:
• organs
• cells
• protoplasts
• tissues derived *in vitro* from roots, stems, leaves, apicals, floral organs

Treatment with various substances increasing the somatic variability (mutagens)

Establishment of the regeneration protocol:
• organogenesis
• embryogenesis

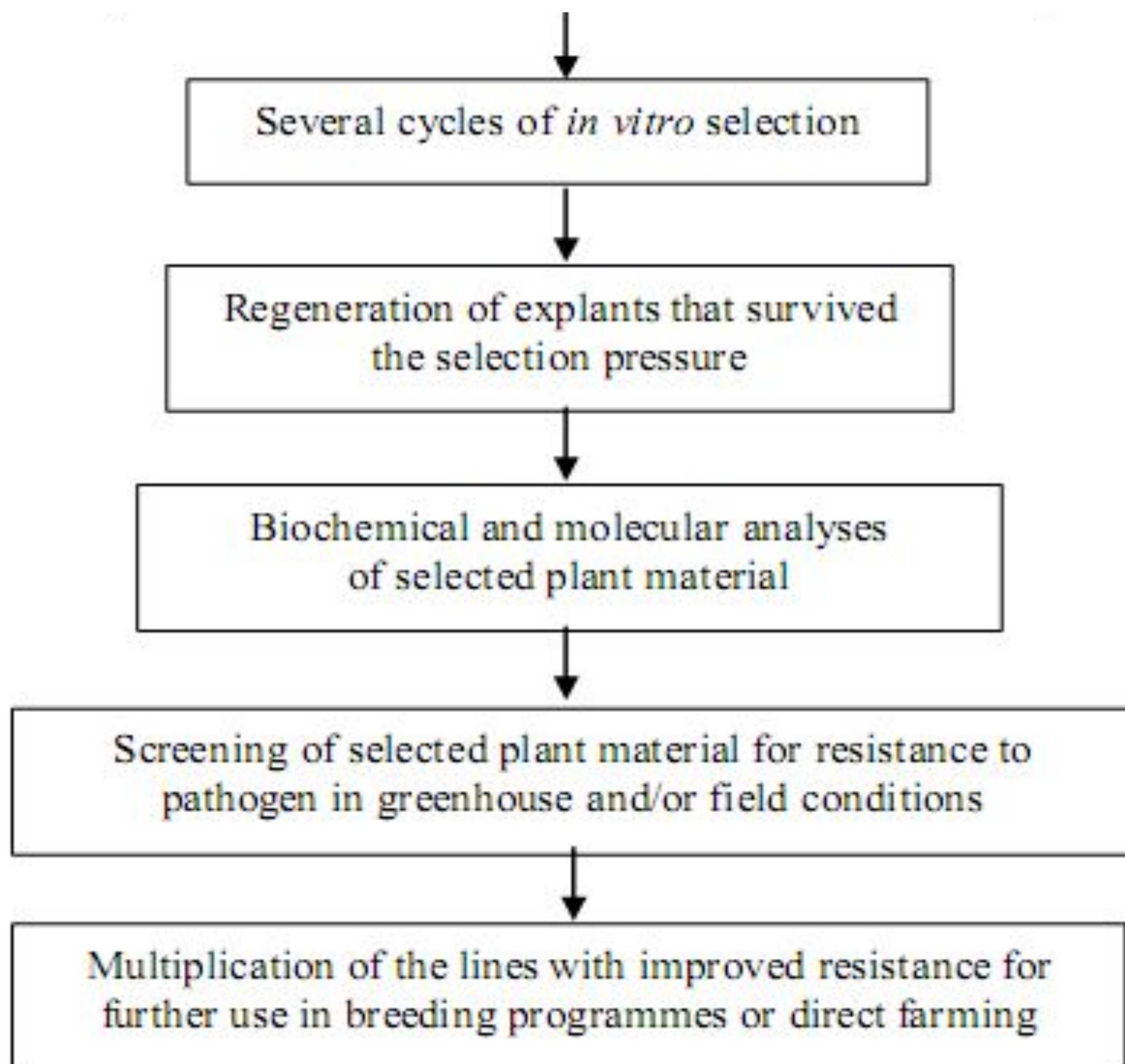
PATHOGEN

Establishment of the isolate culture:
• purification
• propagation

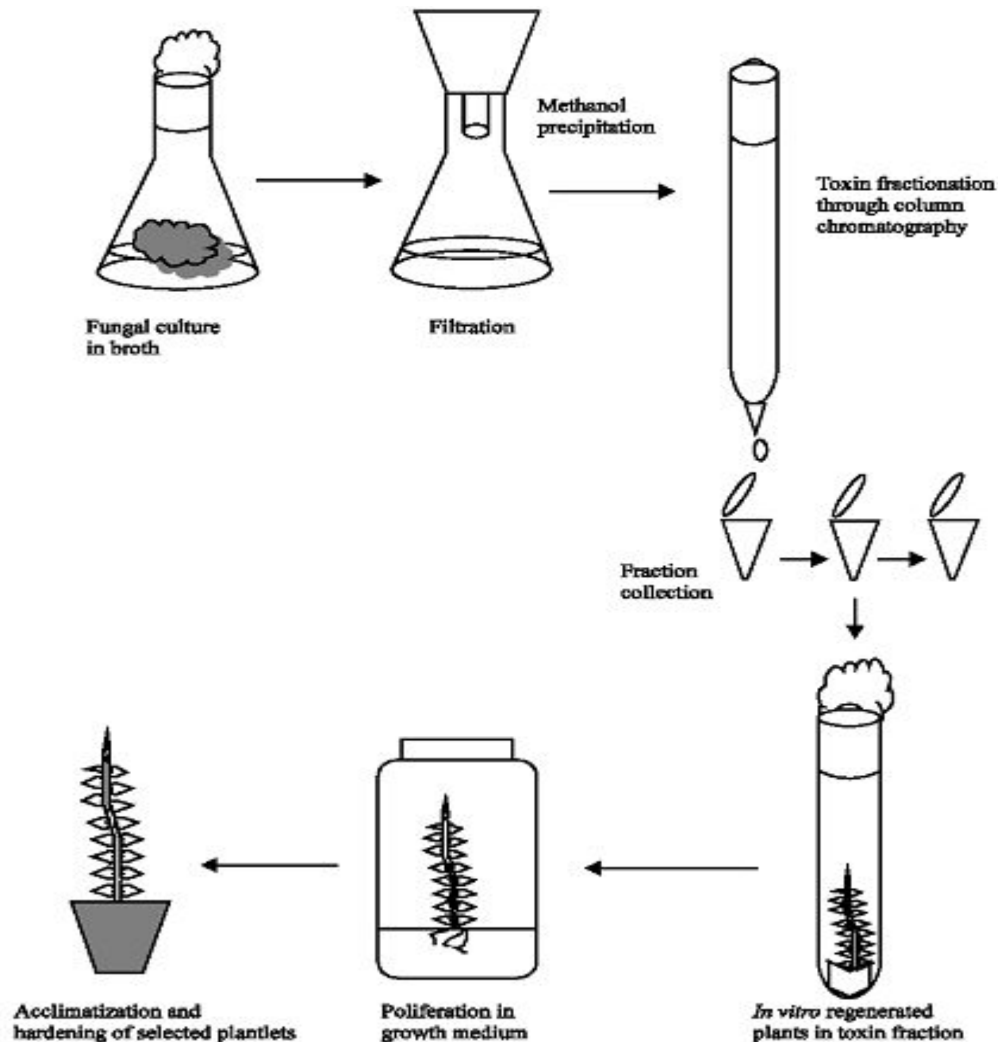
Biological characterisation of isolates:
• virulence
• pathogenicity

Isolation of the selection agents:
• inoculation
• double layer
• culture filtrate
  • crude × purified
  • autoclaved × filtered through millipore
• phytotoxins/pathotoxins
• elicitors

• Establishment of selection concentrations for a particular explant culture
• Establishment of the evaluation method for the assessment of the effect of selection agents in cultures *in vitro*

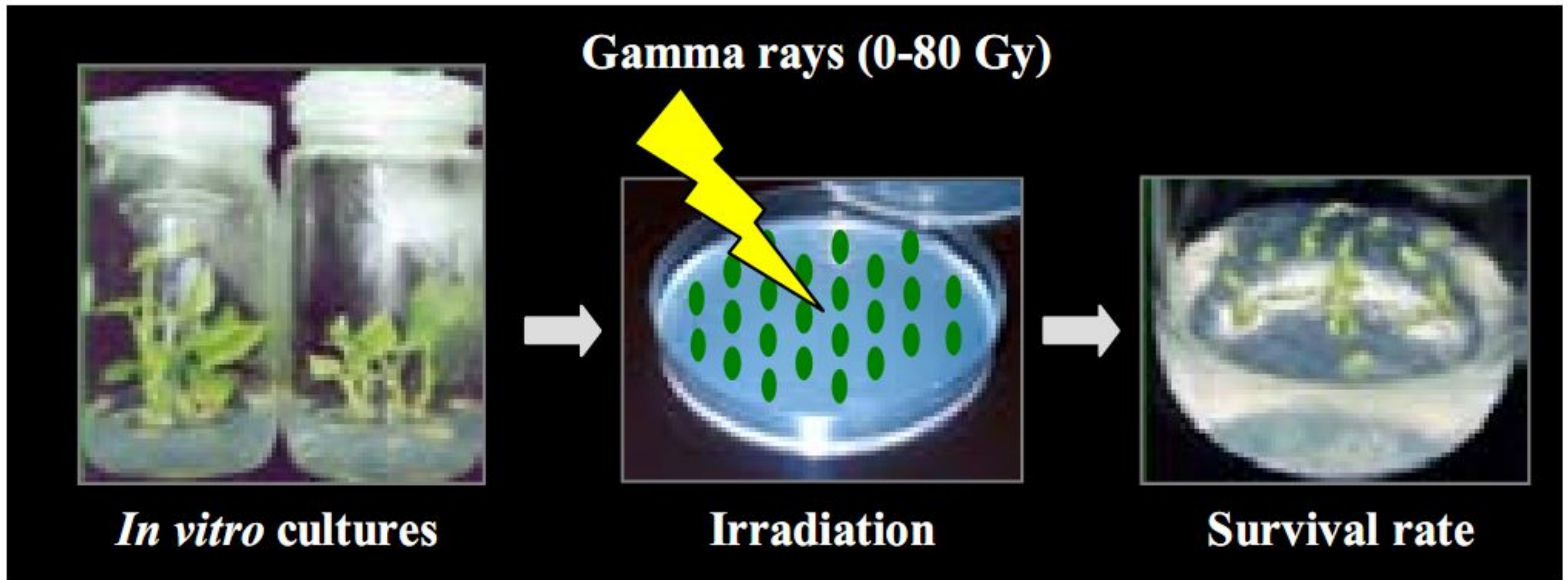

Several cycles of *in vitro* selection
Regeneration of explants that survived the selection pressure
Biochemical and molecular analyses of selected plant material
Screening of selected plant material for resistance to pathogen in greenhouse and/or field conditions
Multiplication of the lines with improved resistance for further use in breeding programmes or direct farming

In-vitro selection for Fungal Diseases resistance



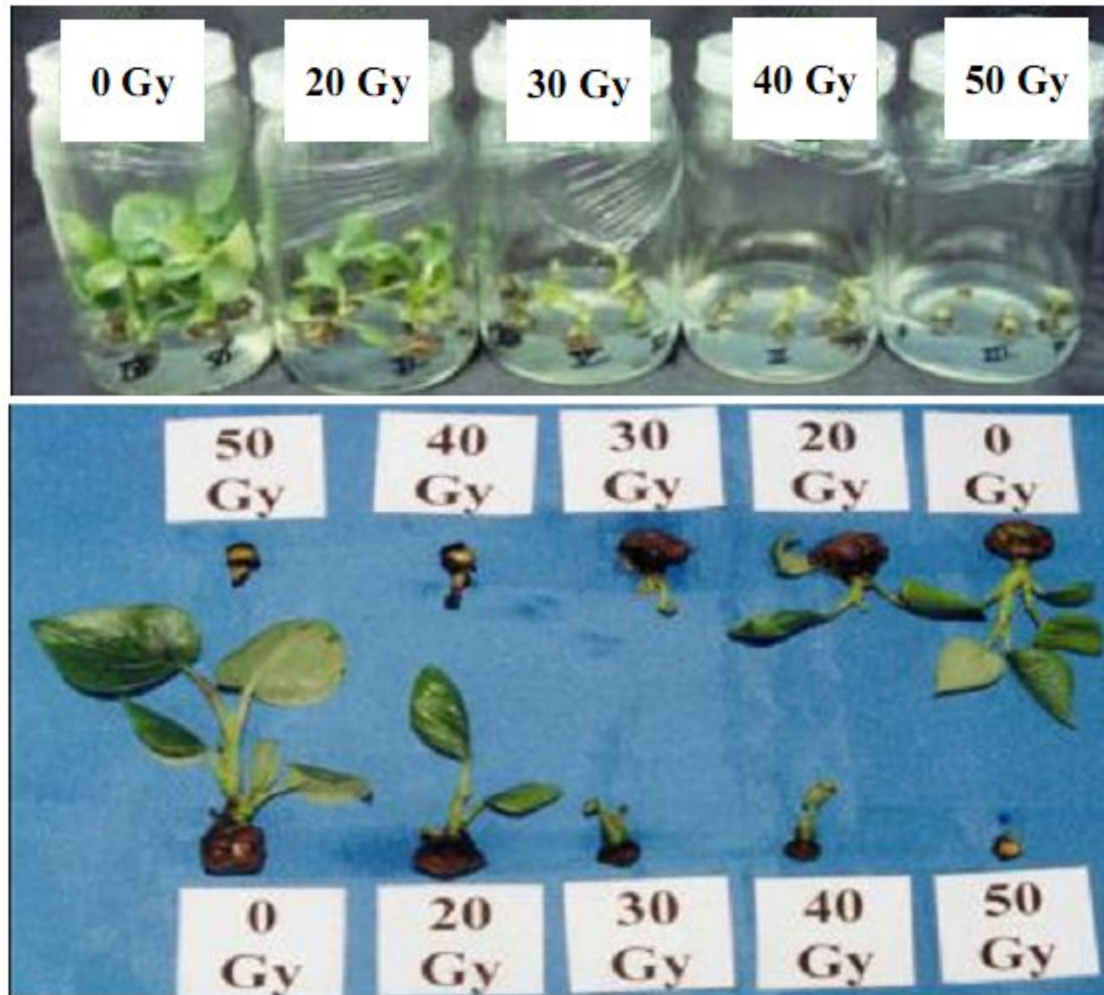
. 1: Schematic representation of steps involved during *in vitro* selection for fungal disease resistant lines

Radio sensitivity Test



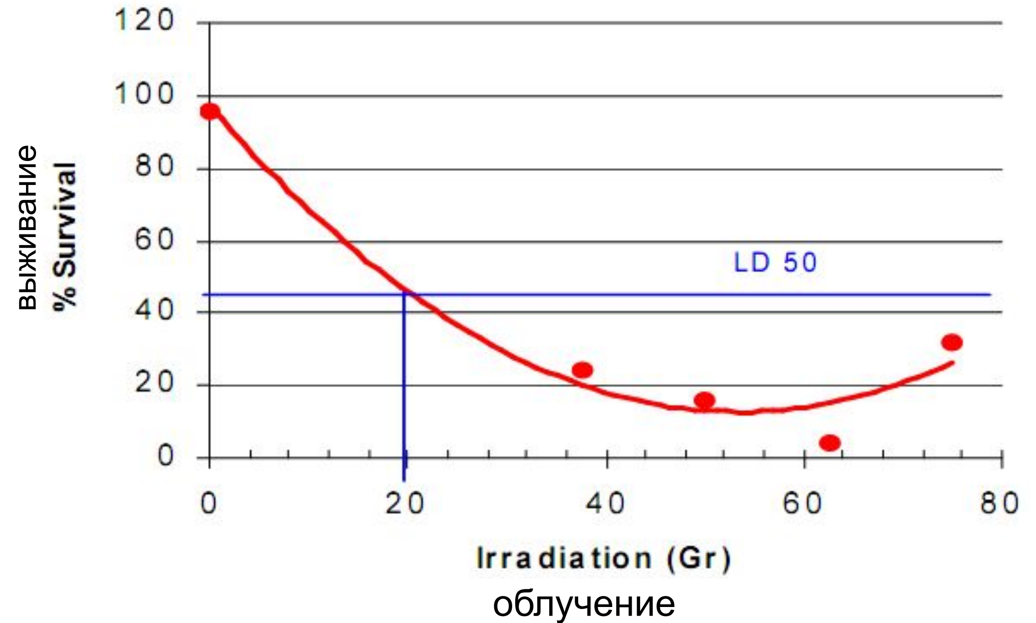
Radio sensitivity test for In vitro buds irradiated with various dose of gamma rays

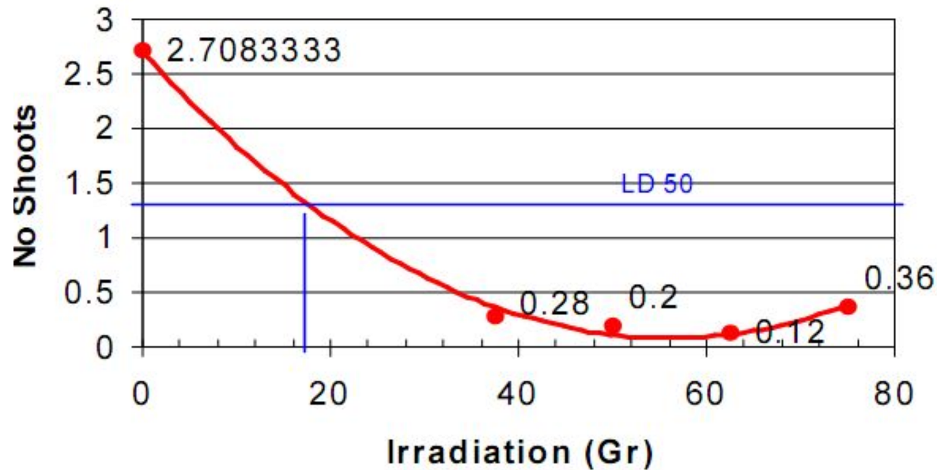
Effect of gamma radiation, doses ranging from 0 to 50 Gy, on *in vitro* buds.



Radiosensitivity Curve or Dose Curve Test

Грей (грэй[1]) (русское обозначение: **Гр**, международное: **Gy**) — единица поглощённой дозы) — единица поглощённой дозы ионизирующего излучения) — единица поглощённой дозы ионизирующего излучения в Международной системе единиц (СИ)[2][2][3]. Поглощённая доза равна одному грею, если в результате поглощения ионизирующего излучения вещество получило один джоуль энергии в расчёте на один килограмм массы.





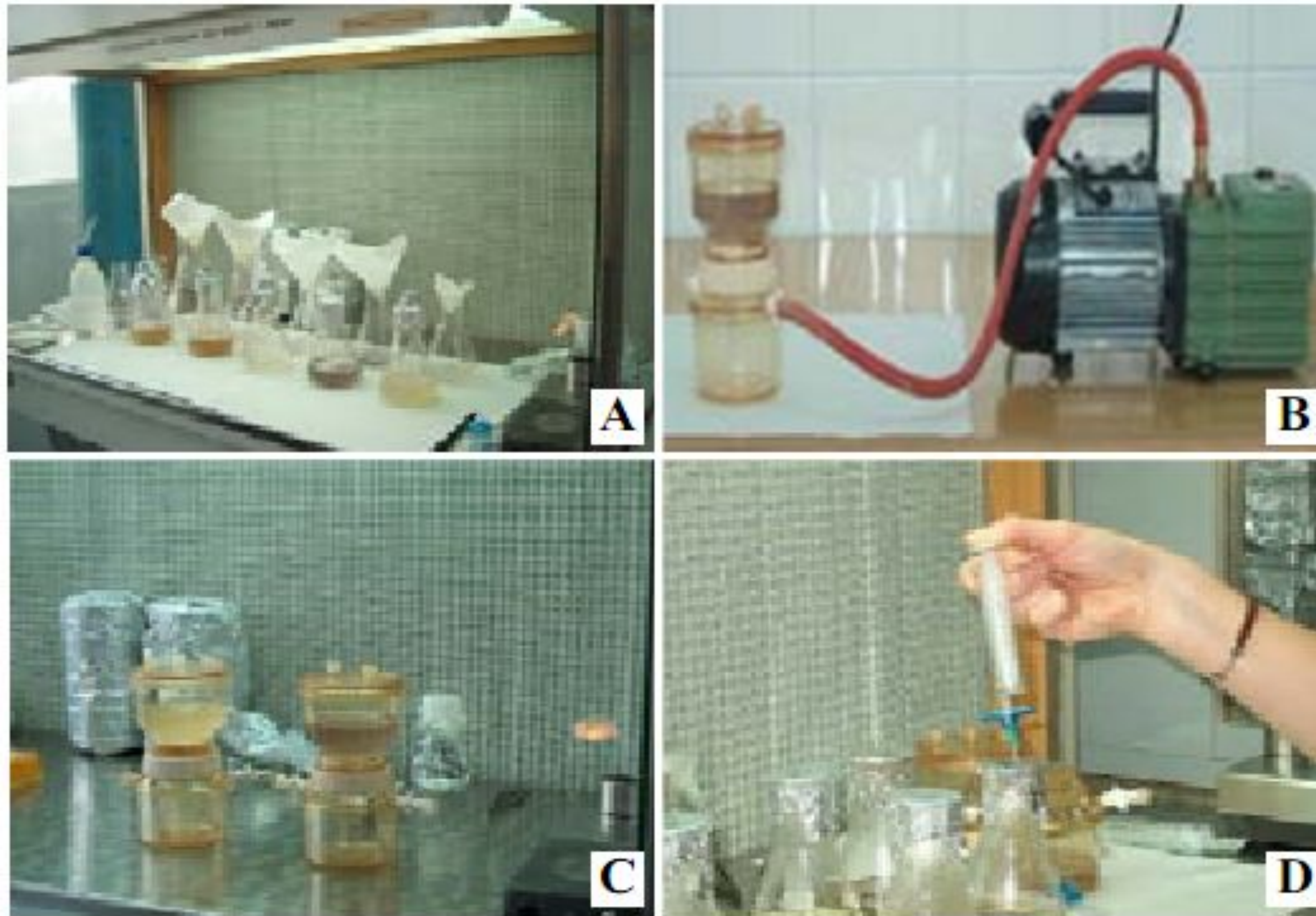


Figure 14.2. The steps of filtrate preparation. (A) Fungal cultures were filtered through filter paper to remove mycelium; (B) Fungal cultures were sterilised with a 0.22 μm Sartorius filter unit with a vacuum for complete removal of fungal cells; (C) After sterilisation; (D) Fungal culture filtrate, which was added to autoclaved basal medium.

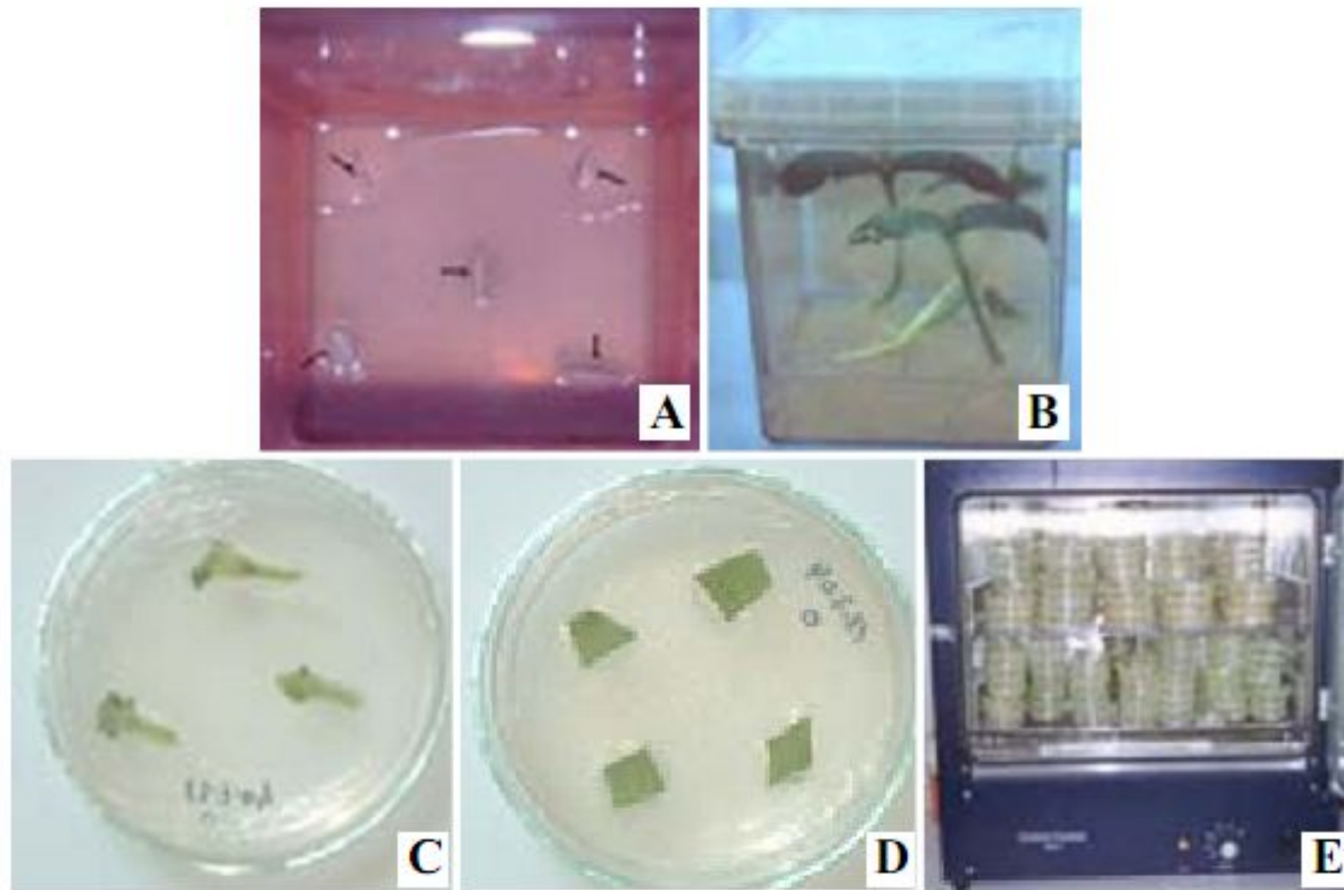
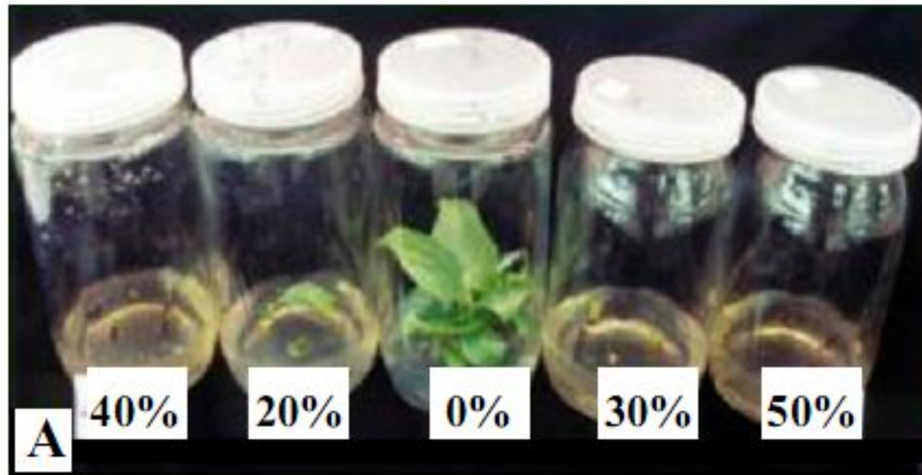
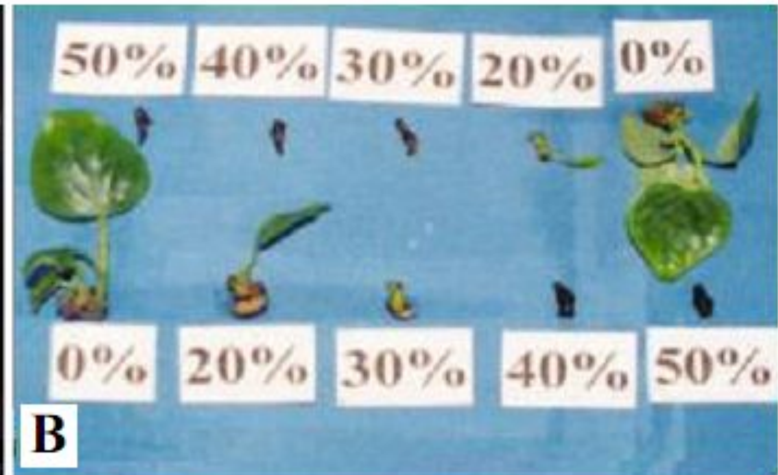
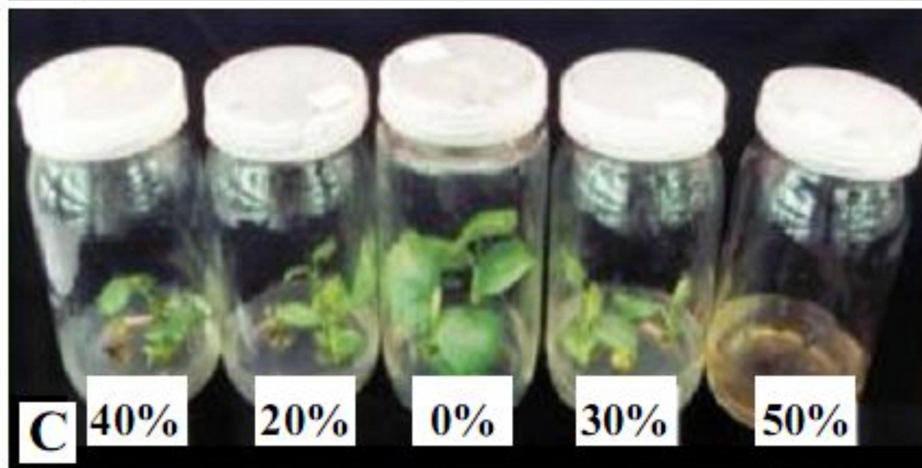


Figure 14.3. The steps for culture initiation. (A) *In vitro* seed culture. Seeds were cultured after removal of the seed cover; (B) *In vitro* plantlet production; (C) Hypocotyls with leaf and cotyledon explants; (D) Cotyledon explants; (E) Incubation period.

Effect of different filtrate concentrations (0, 20, 30, 40 and 50%) as a selective bud multiplication medium. (A-B) Filter sterilisation (FS) after 45 days of vation; (C-D) Double autoclaving (AII) after 45 days of cultivation.

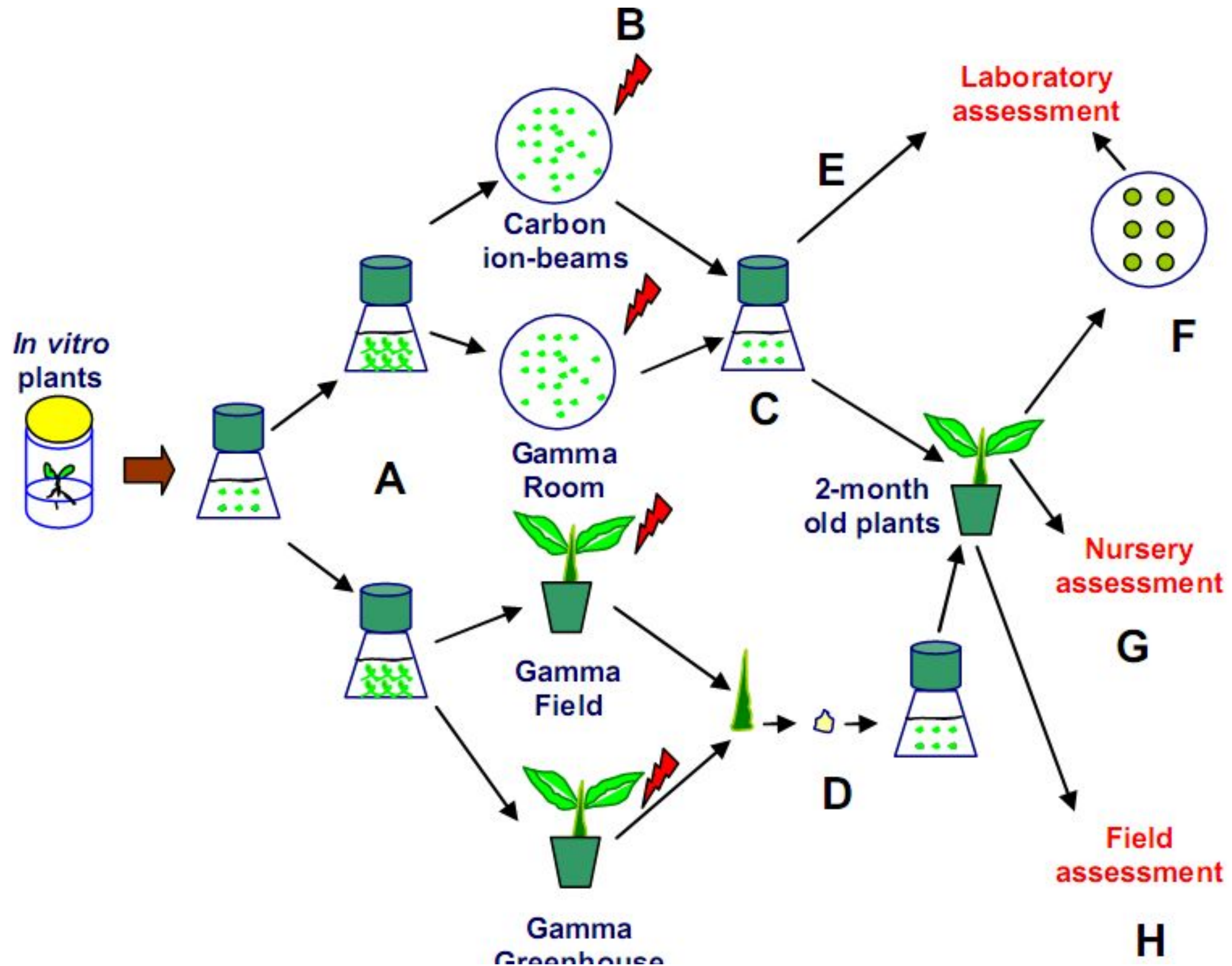




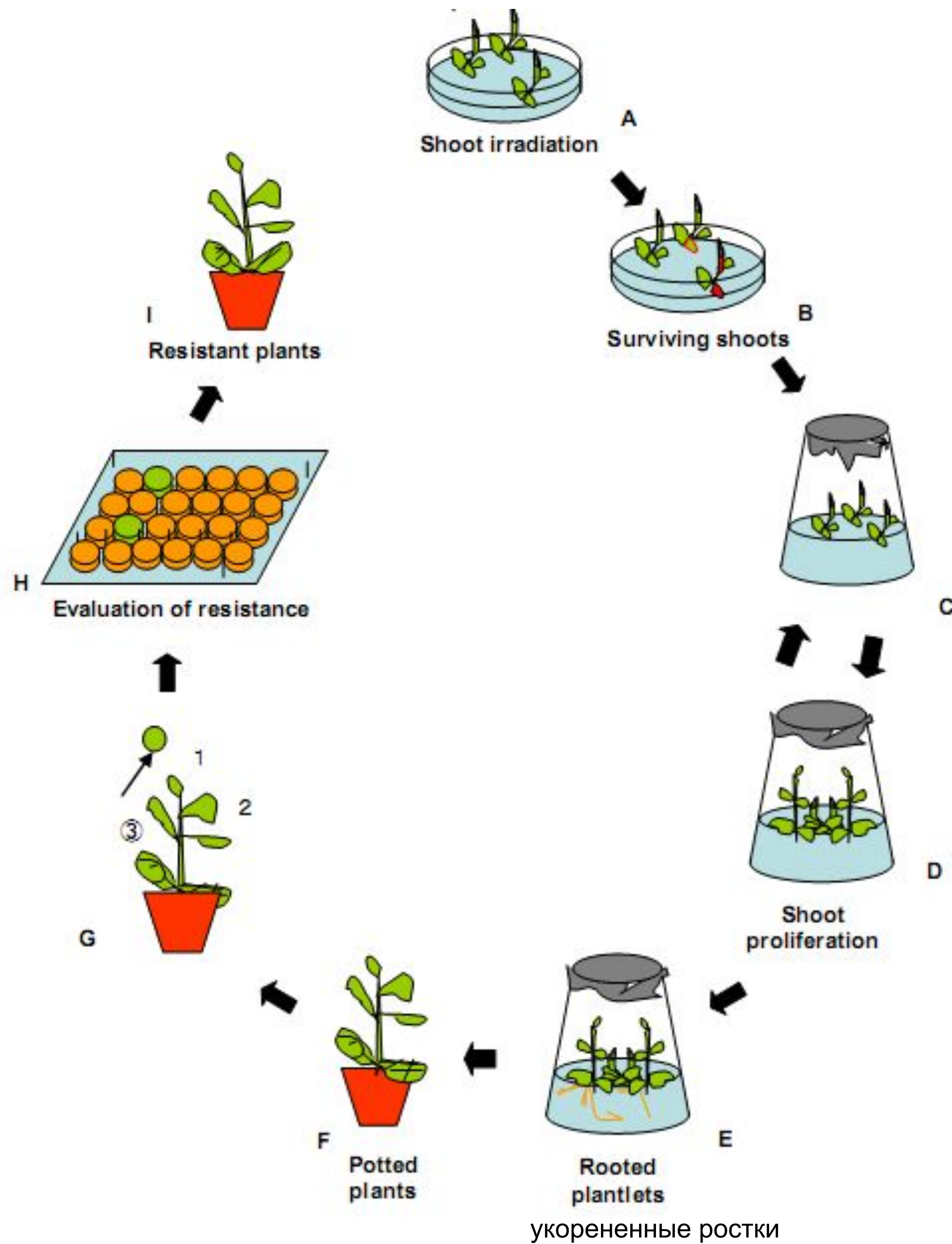


In vitro selection of variants originating form gamma irradiation of buds which survived the culture filtrate of *Fusarium solani*

Screening for Banana sigotaka Leaf spot

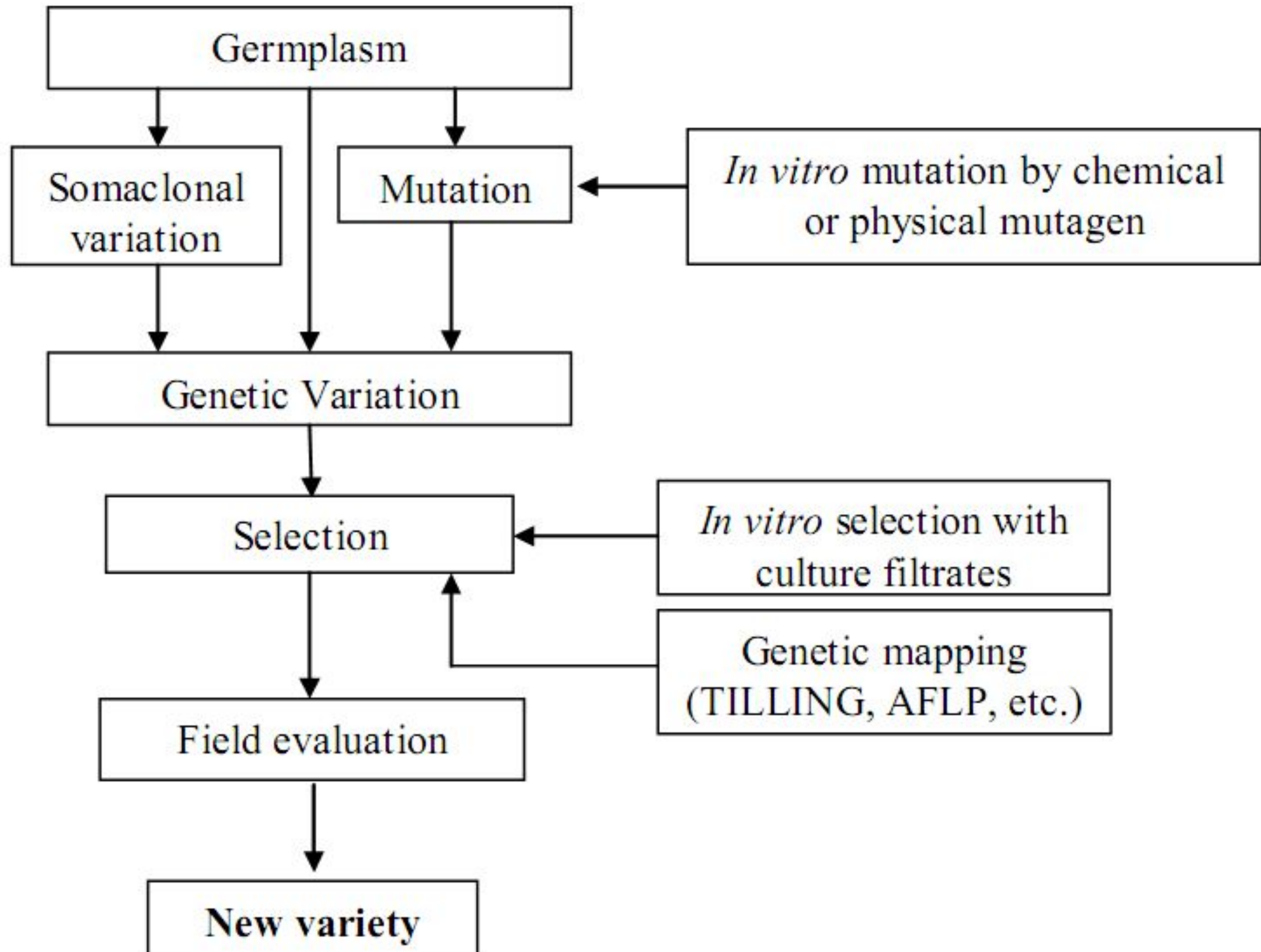


Screening for Alternaria Blotch resistance



укорененные ростки

Plant Breeding Scheme by induced mutations and somaclonal variation



A list of disease resistant plants of various species obtained by *in-vitro* selection

Crop	Pathogen	Selective agent	Selection level	Resistance observed
Barley	*Fusarium spp.*	Fusaric acid	Callus	Increased resistance
Barley	*Helminthosporium sativum*	Crude toxin	Callus	Resistance
Maize	*Helminthosporium maydis*	HmT toxin	Callus	Resistance
Oats	*Helminthosporium victoriae*	Victorin	Callus	Resistance to victorin
Potato	*Phytophthora infestans*	Culture filtrate	Callus	Reduced lesion size
Rape	*Phoma lingam*	Culture filtrate	Suspension cells	Increased resistance
Rape	*Alternaria brassicicola*	Partial culture filtrate	Secondary embryoids	Increased resistance
Rice	*Helminthosporium oryzae*	Crude toxin	Callus	Increased resistance
Rice	*Xanthomonas oryzae*	Bacterial cells	Callus	Resistance
Sugarcane	*Helminthosporium sacchari*	Toxin	Callus	Increased resistance
Tobacco	*Pseudomonas syringae pv. tabaci*	Crude toxin	Callus	Resistance
Tobacco	*Tobacco mosaic* Virus	Virus	Callus from infected tissue	Reduced virus
Tomato	*Fusarium oxysporum f.sp. Lycopersici*	Culture filtrate	Callus	Tolerance to culture
Wheat	*Helminthosporium sativum*	Crude toxin	Callus	Resistance

Thank You