“PLANT BIOTECHNOLOGY BASIS OF PLANT PROTECTION”

Lecture works
For preparing the specialists 6.090105 “Plant Protection”

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Educational publication
List of lecture works
For preparing the specialists 6.090105 “Plant Protection”

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CONTENT
THEME 1. INTRODUCTION. PLANT BIOTECHNOLOGY AS A MODERN SCIENCE ....................................................................................................................... 4
THEME 2. CLONAL MICROPROPAGATION ................................................................................................. 7
THEME 3. CULTIVATION OF EMBRYO. FERTILIZATION IN VITRO. SEXUAL REPRODUCTION OF PLANTS ........................................................................................................... 11
THEME 4. PLANT CELL BREEDING ........................................................................................................... 17
THEME 5. ISOLATED PROTOPLAST CULTURE AND SOMATIC HYBRIDIZATION OF PLANTS ................................................................................................................ 22
THEME 6. GENETIC ENGINEERING OF PLANTS AND THE INTRODUCTION OF ITS ACHIEVEMENTS IN THE PRODUCTION .......................................................................................... 32
THEME 7. CRYOPRESERVATION METHODS AND PLANT CELLS BANKS - THE BASIS OF NEW BIOTECHNOLOGIES ............................................................................................................. 39
THEME 8. RECEPTION BIOLOGICALLY ACTIVE SUBSTANCES .................................................. 41
THEME 9. PROBLEMS OF ECOLOGICAL SAFETY .................................................................................. 47
THEME 1. INTRODUCTION. PLANT BIOTECHNOLOGY AS A MODERN SCIENCE

Plan
1. Introduction. Plant biotechnology.
2. Object, objectives, methods of plant biotechnology.
3. History of biotechnology.
4. Basic concepts of plant biotechnology.

Fundamental researches in physiology, genetics, biochemistry and molecular biology of plants, carried out of using the methods of cultivation in vitro in aseptic conditions, and using of its results in industrial production for the last 30 years have shaped and developed the self-discipline - **biotechnology**.

**Biotechnology** is a field of applied biology that involves the use of living organisms and bioprocesses in different fields of humans’ activity such as engineering, technology, medicine and agriculture.

**Plant Biotechnology** is a collection of technologies that including a using of biological processes of living cells, macro-and micromolecules with the purpose of obtaining a specified products.

The main objective of biotechnology research:
Improve of an existing varieties and obtain a new varieties, species, hybrids of high forms of plants with improved indicators of quality products and resistance to diseases and pests (useful characteristics to humans).

There are three ways in modern plant biotechnology:
- Technologies, based on the using of cell cultures, tissues and organs of plants;
- DNA technologies (molecular-genetic methods for analysis of plants);
- Obtaining of transgenic plants.

![Pic. 1. Branches of Biotechnology](image)

**Objective biotechnology**
- creation of new biologically active substances and drugs for medicine and effective prevention, diagnosis and treatment of humans and animals;
- creation of methods of protection from diseases and pests, bacterial fertilizers, plant growth regulators, resistant to adverse environmental factors varieties
and hybrids;
- creation of a valuable feed additives and biologically active substances for using in productivity of animals in order to increase it;
- creation of a new technologies for production of valuable products for using in food, microbiological and other industries;
- creation of a waste and environmentally safely recycling technologies and bioconversion of agricultural, industrial, municipal waste to produce energy, high-quality organic fertilizer, protein and vitamin feed additives;
- improvement and optimization of equipment for biotechnological processes in order to maximize the yield of products;
- increase technical and economic indicators of biotechnological processes compared to existing ones.

**Pic. 2. Objects in Biotechnology**

**Historical stages of development of plants biotechnology:**

**Stage I:**
- End XIX - beginning XX centuries. German scientist **G. Rehinher** (1893), received and studied callus formation in sugar beet roots;
- **H.Haberlandt**, substantiated the idea of the possibility of growing some plant tissues on artificial nutrient media. Detailed development of this method was made in the 30's by american **Phillip White** and frenchman **Roger Gotra**.
Stage II - 1940-1960:

- **1949** - F. Kalinin began work on the cultivation of isolated tissues and organs of plants in Ukraine.

- **1957** - F. Skuh and K. Miller (USA) demonstrated the possibility of obtaining the roots and shoots of callus tissue with the influence of cytokinins and auxins. Zh. Morel (France) found gibberellin effect on proliferation of meristem, its differentiation and development of the whole plant.

- **1957** - R. Butenko launched physiological and biochemical studies of culturing isolated cells and tissues in regeneration.

- **1958-1959** - F. Stuart, J. Reinhardt experimentally proved the possibility of somatic cells to form embryo structure (somatic embryos (non sexual)).

Stage III: development of a new areas of plant biotechnology - somatic hybridization and genetic engineering:

- **1970** - the beginning of work on the cultivation of isolated tissues and organs in the Institute of Botany of the Academy of Sciences of the USSR. In the Nikitsky botanical garden started work on culturing isolated embryos of fruit crops (A. Zdruykovska-Richter).

- **1971-1975** - K. Kao (Canada), E. Kokinh (UK), E. Takebe (Japan) began work based on isolation and fused protoplasts.

- **1980** - Y. Gleba, V. Sydorov, M. Piven, I. Komarnicki, T. Pasternak (Ukraine) began a series of works with isolated protoplasts. Received somatic hybrids and cybrids, started first work on genetic transformation.

- **1990** - E. Piruzyan, V. Andrianov, V. Mett, I. Stehin (USSR) - start of working on with cloning genes and for initial transgenic plants.

- **1994** - In the USA reported the first transgenic food use - tomato "Fleyvor-Savior" characterized by longer post-harvest storage.

- **1999-2000** - the number of transgenic plant varieties in the world reaches over 120 and they occupy over 40 million hectares of farmland.

- **2002-2005** - area under transgenic crops in the world are more than 57 million hectares.

The concepts and terms

- **Apical domination** - growth inhibition of lateral buds of shoot with the presence of apical (terminal) meristem.

- **Dedifferentiation** - transfer of specialized cells that do not divide, to proliferate, which leads to the loss of most of the signs of specialization.

- **Diploid** - a nuclear, a cell, an organism, characterized by a double set of homologous chromosomes, cast by number, characteristic of this species.

- **Differentiation** - the complex of processes that lead to differences between the cells, as well as between parent cells, the state of specialization of cells that distinguishes them from others.

- **Explantat** - a piece of tissue or organ that used to grow in vitro independently or for the primary callus.

- **Isolated protoplast** - plant cells devoid of cell walls by enzymatic destruction.
or mechanically.

- **In vitro** - cultivation of living material "in glass", on artificial nutrient media under aseptic conditions.
- **Inoculum** (transplant) - part of the suspension (callus) cultures used for subculture.
- **Callus** – tissue arising from dedifferentiation and disorganized proliferation of cells on the surface of the wound. In the culture in vitro for the first subculture called primary callus.
- **Organogenesis** - the process of arising de novo in the mass of callus cells that grow disorganized, the process of arising primordia of organs (roots and shoots).
- **Rhzogenesis** - the formation of root callus cells.
- **Recessive** - no phenotypic expression of one allele in heterozygous individuals, i.e., individuals with two different alleles of a single gene.
- **Subculture of transplant** – is transfer of explants, callus, plant regeneration to the other culture vessel on fresh nutrient medium.
- **Totipotency** - property of somatic plant cells to realize their development potential, so realized omnipotented nucleus to form an entire organism; the ability of plant cells phenotypically implement the genetic information encoded in the DNA of the nucleus.

**THEME 2. CLONAL MICROPROPAGATION**

Plan:
1. Types and stages of clonal micropropagation
2. Preparation of virus – free planting material
3. The practical significance of the method of clonal micropropagation

**Clonal micropropagation** - using of techniques in vitro for quickly obtaining unsexual plants identical to the original.

**Preferences of microclonal breeding of plants comparatively with traditional methods are:**

- significantly higher rates of reproduction, 105-106 clones per year, traditionally during the same period from one plant receiving 5-100;
- miniaturization of process that saves space occupied by uterine and propagated plants;
- recovery of the original plant material from nematodes, bacteria, viruses.

Initiation - the initial stage.

Proliferation - the formation of new cells and tissues by multiplication of existing cells.
The basic method - activation of already existing in the plant meristem.

2 ways:
- removal of the apical meristem of a stem and further micro cutting (stalk) of shoots in vitro on non-hormonal nutrient medium;
- adding to the culture medium substances with cytokinins type of action (C), which induce the growth of multiple axillary shoots.

The second method - induction of occurrence of buds or embryos
- Based on the ability of isolated parts of the plant under favorable conditions of culture (nutrient) medium to recover missing plant organs and thus regenerate the whole plant.
- In this way, were multiplied many representatives of the lily family, tomatoes, and other woody plants.

Stages of microclonal reproduction (clonal micropropagation):
- Choosing of the donor-plant, isolating of explants and getting a good-growing sterile culture;
- Micropropagation, when it reaches a maximum number of meristematic clones;
- Rooting of propagated shoots with further adaptation to soil conditions;
Growing plants in a greenhouse and prepare them for sale or planting in the field.

Study of the process of morphogenesis in vitro at all levels of the organization (from individual cells to the tips of shoots) were led to the creation of microclonal breeding plants technology that has largely become commercialized.

The term "clone" (from the Greek. – shoot) was proposed by Weber in 1903 for vegetative propagated plants. It is assumed that shoots of plant reproduces asexually, isn’t the individual in the usual sense.

They only parts (clones) of the individual parent, identical to her and each other. So microclonal reproduction - is using in vitro technology for fast unsexual obtaining of identical plants.

Advantages of microclonal plant propagation compared with traditional methods are: significantly higher rates of reproduction, the estimated reach 105-106 clones per year, whereas traditionally for the same period of one plant receiving 5-100; miniaturization of the process, which saves space occupied uterine and propagated plants; improving of plant material against nematodes, bacteria, viruses.

A major advantage of the technology is the fact that in vitro often make a process of rhizogenesis those plants do not reproduce or badly reproduce by normally way. It is also important that the planting material obtained in this way is genetically identical to the original plant, as formed from the meristem of somatic plant cells.

At the same time the sexual reproduction of plants descendant growing of zygotes formed by the merger of two gametes of different species.

This zygote contains genes as the parent, and the parent body because offspring that occurs during sexual reproduction not identical to the parent form and carries the hereditary characteristics of both parents.

**1. Types and main stages of microclonal reproduction**

Depending on the nature of morphogenetic processes (methods for producing regenerated plants) in tissue culture distinguish types (methods) of microclonal reproduction, which are based on the differences obtaining plants (with the already existing plant in vivo newly initials).

Accordingly, there will be a little different plant-derived regenerates.

The first type of plants formed by activation of existing intact plant meristem (stem apex, axillary buds and dormant stems), so by direct morphogenesis. These plants regenerated from meristem is genetically identical to the parent form because apex in a culture largely genetically stable.

The second type of plants formed by the induction of renal origin or embryos. These plants derived from a specialized and callus cells, which have genetic variability, often different from the parent. Thus, this method applies only to those plants which callus is different in genetic stability or variability between plants-regenerate is not exceed the level of natural variability.

Buds or embryos may arise by:

- directly from specialized tissue explants;
- the primary callus, formed by explants cells;
- subculture of callus tissue or cell suspension culture.

It is believed that the formation of plant-regenerates directly from explants (by
activating of existing meristem, formation of buds or embryos) describes the direct morphogenesis.

Formation of plant-regenerates from primary callus or subculture callus is characteristic for indirect morphogenesis.

In essence microclonal propagation similar to vegetative type such as plant propagation, with the only difference that it occurs conditions in vitro, where cells isolated tissues can get quite a lot of new plants.

Obligatory condition of microclonal propagation is identification of derived plant material to original parent plant.

More recently, this method is regarded as an opportunity to accelerate the cloning of plant species that reproduce vegetatively as well as an auxiliary method of healing plants against viruses.

However, the results of some experiments have shown that the value of this method increases significantly for clonal selection of plants and vegetatively propagated, cryo-preservation of valuable source material and especially the study of individual genetics of crops that reproduce by seeds.

The ability to form a large number (several million or more) in somatic embryos in vitro, which is considered as one of the ways of practical using the method of microclonal propagation is used to develop the technology and continuous mass of artificial seeds.

It can convert some heterosis breeding farm cultures for more effective and purposeful. Moreover, the method of microclonal breeding successfully used in the creation of synthetic varieties.

Currently, the number of species that can be cloned in vitro, more than 1 thousand.

More than 100 species, this method is a real commercial applications. The main advantage of the method of microclonal reproduction compared to other
classical methods - the highest multiplication factor. If the usual way (cuttings, bulbs, rhizomes) from one plant can get 10-100 plants per year, by microclonal propagation methods can be increased from 50 thousand to 1 million.

Therefore, to provide the required amount of plant any breeding program can be used by individual and super elite plants. This is the high efficiency of the method. Millions of plants can be grown on a small laboratory area (150-200 m²), with a lower cost of electricity and labor.

*Factors that affect the propagation microclonal*

The most important point is the selection of parent plants and explants, and sterilization of the source material.

*Select the parent plant.* It is advisable to source plants are not damaged by fungal, bacterial and viral diseases. Bulbs, roots and tubers before entering the culture *in vitro* pretreated with curly or low temperatures for different time - from several hours to several months.

*Select of explants.* To maximize genetic stability of cloned material and to avoid the appearance of abnormal plants as source using explants of young weakly differentiated tissue. For this is most suitable tips of stems, lateral (axillary) buds, germs and other meristematic tissue. Can use the young leaves, petioles, buds, scales, bottom bulbs.

*Sterilization of source material.* For disinfection of initial explants using conventional techniques.

However, often the internal contamination source explants is much stronger than the surface infection, so the explants pre-treated with fungicides and antibiotics against fungal and bacterial infections. Good results are obtained by processing the plant material sodium benzoate.

Depending on the type of plant used both solid and liquid culture media, horizontal or vertical position of explants in a nutrient medium should also establish optimal ratio of explants and the number of medium, optimal lighting conditions.

**THEME 3. CULTIVATION OF EMBRYO. FERTILIZATION IN VITRO. SEXUAL REPRODUCTION OF PLANTS**

Reproduction of plants associated with the alternation of two phases, or generations in the life cycle of plants - generation of saprophytes and gametophyte generation.

Sporophytes of higher plants - is the plant we usually see. Generation of saprophytes begins with egg fertilization and includes the stages of:
- seed;
- seedlings;
- fully-formed plant with flowers (Pic. 6).
Pic. 6. The alternation of gametophyte and saprophytes generations by sexual reproduction of plants, single germ, double ring labeled physical cells. (by Schmalz, 1973).

Flowers of sporophyte form spores of two types:
- Men, formed in the anthers and called microspores;
- Women, formed in the ovary is called megaspores (Pic. 7).

Microsporogenesis  Megasporogenesis

<table>
<thead>
<tr>
<th>Parent cell of microspores</th>
<th>Parent cell of microspores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphase I of meiosis</td>
<td>Metaphase II of meiosis</td>
</tr>
<tr>
<td>Microspores tetrads</td>
<td>Degeneration of three microspores</td>
</tr>
<tr>
<td></td>
<td>Microspores tetrads</td>
</tr>
</tbody>
</table>
Pic. 7. Comparative scheme of male and female gametes in flowering plants.
(by Cartel etc., 1999).

Sporophyte - diploid (2n), and microspores and megaspores - haploid (n).
From microspore in anther is developing a pollen grain (pollen). After cracking of anther a matured pollen reaches on the stigmas pistil and there germinates to form a pollen tube with two generative nuclei (sperm) which grows to the embryo sac in the seed germ. In the pollen tube never produced more than three nuclei (Pic. 8).

Pic. 8. Development of pollen (from the mother pollen cell to the pollen tube):
A – anther; B - anther cross-section; C - the stage of formation of tetrads of mother pollen cell by process of meiosis; D - four microspores; E - pollen grain; F - germination of pollen grain in the pollen tube; 1 – anther; 2 - pollen sac; 3 – stamen thread; 4 – tapetum; 5 - pollen parent cell; 6 – diad; 7 – tetrad; 8 - generative cell; 9 - vegetative nucleus; 10 - old pollen grain; 11 - pollen tube; 12 - generative nucleus (sperm) (by Briggs, Knowles, 1983).

Vegetative nucleus determines the germination of pollen tubes toward the pestle base and generative nucleus (sperm) droop in the embryo sac, one of which fertilizes the egg nucleus, the other - a secondary nucleus of the embryo sac from which the crops growing in the endosperm.

Generative nucleus – is a male gametes. Female gametophyte is called the
embryo sac, formed from megaspore. Megaspore divided three times because the embryo sac has eight nuclei. One of them is the nucleus of the egg and the other two are merged, forming a polar core. The core of the egg and the polar nucleus surrounded by cytoplasm and cell membrane, resulting in the formation of the egg and the endosperm mother cell.

Pic. 9. Schematic view of the fertilization process.

The number of chromosomes in the sperm and egg are haploid. The merger of the egg nucleus and sperm nucleus restored the normal diploid number of chromosomes are characteristic for this type of plant. After fertilization of the secondary (diploid) nucleus of the embryo sac is formed endosperm nucleus containing triploid set of chromosomes (Pic. 9).

After fertilization an egg is called a zygote, it divides and develops in the embryo (2n), and from the maternal endosperm cell developing endosperm (3n). Endosperm may terminate its development in the early stages, while the seed consists of the shell and the embryo.

Sexual reproduction is characterized by a complex system of adaptive mechanisms, the main ones are:
- formation and development of male and female gametophyte, resulting in formation of male and female gametes;
- gametes fusion of opposite sex;
- integration of nuclei;
- integration of homologous chromosomes in meiosis, followed by recombination of hereditary factors in the formation of new gametes.

For irregular types of sexual reproduction includes parthenogenesis, gynogenesis, androgensis, merogenesis.

Parthenogenesis - one of the ways of sexual reproduction in which the egg is under the influence of internal or external factors begins to divide and develop into normal embryos without sperm.
Pic. 10. Types of sexual reproduction of plants:
1 – normal fertilization, 2 – parthenogenesis, 3 – gynogenesis, 4 – androgenesis

(Lobashov, 1975)

Parthenogenesis depending on the set of chromosomes in a cell that divides is generative (or haploid) and somatic (diploid or polyploid).

The development of the embryo from the egg entirely by the female nucleus called gynogenesis. Spermatozoons in this case needs only to activate the nucleus: it penetrates the egg cytoplasm but not fertilizes the nucleus (so-called fake fertilization - pseudogamy).

The opposite of gynogenesis is androgenesis in which embryo development is carried out by one or two nuclei of sperm that penetrated the egg, the nucleus of which is dissolved (elimination). Embryo development is carried out by a male nuclei without women.

Meroogenesis - artificially caused by the initial development of embryo from the egg cytoplasm without the female nucleus.

**INCOMPATIBILITY AND ITS GENETIC BASIS**

Self-incompatibility - the inability to form seeds with process of self-pollination.

Incompatibility systems are divided into two groups:
- Heteromorphous;
- Homomorphous;
  - Homomorphous divided:
    - Gametophytes types of incompatibility;
    - Sporophytes types of incompatibility.
  - Heteromorphous incompatibility system determined by different lengths of stamens and pistils.

Gametophytes incompatibility system caused by special hereditary sterile factors. Functional ability of pollen is determined by its genotype, rather than genotype plants (sporophytes).

Sporophytes incompatibility system due to dichogamy – is simultaneously
maturation of male and female gametophyte.

Dichogamy divides into two types:
- protogyny - when stigmas can take pollen before maturation;
- protandry - when pollen is mature and emptied before being formed into a secret pistil stigmas.

These systems of incompatibility can be represented by the scheme (Pic. 10).

![Incompatibility Diagram](image)

Pic. 11. Basic scheme of incompatibility

Cytological studies of incompatibility suggests that its causes may be:
- inhibition of pollen germination;
- pollen germinates normally, but inhibited the growth of pollen tubes in the column;
- pollen tubes grow normally and gametes reach the germ seed, but the seed isn’t formed.

**CULTURE OF ISOLATED EMBRYOS (EMBRYO CULTURE)**

The method of cultivation of isolated on nutrient media (embryo culture) launched in 1904 by Hanning.

Cultivation *in vitro* involves growing them in specially selected media under aseptic conditions, previously isolated from mature seeds from immature seeds.

In the first case, embryos differentiated, in the second they are in different phases of development.

**Stratification** - performance of low temperatures.

**Scarification** – damaged of hard seed skin to enhance the ability to swell to speed up germination.

Mechanisms of dormancy and germination depend on:
- lighting;
- storage temperature;
- the presence of endogenous inhibitors.

During the culturing of isolated embryos from mature seeds usually use simple mineral-sucrose medium without vitamins and growth regulators as biologically active compounds often trigger abnormal forms of spore ongoing individual organs and appears callus.

Embryos isolated from mature seeds after swelling:
- seeds are sterilized;
- sometimes additionally burn in the flame of alcohol lamp;
- in sterile conditions produce embryos.

The most important requirement with isolated embryos is to choose the medium that can support their growth and development.

Simpler method is sterile plants method for hybrid seed plants from crosses that form viable seedlings. As a result of interspecific hybridization seed is formed, step by dying on stage deployment cotyledons. Growing seeds in vitro on a simple mineral medium with 1% solution of sucrose leads to the formation of adult hybrid plants. In the conditions in vitro primary root of sprouted seeds can suppress growth and become extinct, but even without roots sprout starts to grow. After regrowth of plant roots planted in the ground.

The main advantages of this method is that the composition of the nutrient solution simpler than in the case of culturing immature embryos.

Embryo culture method provides the selection of plants resistant to diseases at an early stage of their development, provided that the reaction of adult plant coincides with the reaction of cuttings derived from embryos.

Using the embryo culture method to study the dependence of embryos host plant and the parasite can be cultivated seeds and embryos of some obligate parasites under aseptic conditions in vitro.

FERTILIZATION IN VITRO

Technologically method involves mandatory castration of self-pollinating plants several days before blooming buds, followed by isolation of parchment or gauze insulators.

The day before the opening of buds cut a few seconds to maintain 70% non-ethanol and sterilized for 10 min chlorine water. Then with tweezers buds removed a pestle, or ovary, the wall of which is cut to expose the beginnings of seed. Sometimes cuts pieces of the placenta from the seed rudiments.

Isolated flower components transferred to the culture medium, and 2-3 days for them add pre-sterilized pollen of plants that want to fertilize the seed germ. Pollen and UV sterilized for about 30 minutes, or collected in sterile boxes with still unsolved buds that before this pre-sterilized. Cultured pollen germinates on agar-agar environment through pollen tube micropyle seed germ get into the embryo sac and fertilize the egg. Fertilized seed germ grows in size. A few days later produced seeds.

The resulting plants are well developed and form flowers, giving male and female spores.

THEME 4. PLANT CELL BREEDING

One of the important trends in plant biotechnology is cell breeding with the breeding of cell lines and plants with new hereditary traits occurs at the level of cells cultured in vitro. The main objective of research on cell selection is to obtain the help of modern methods of molecular and cell biology of a wide spectrum of mutations in plants, selection of their most valuable and after physiological and genetic study of their use in breeding work to create new varieties. Preparation of plants sampled in a selective mutant cells is possible due to the unique properties of the plant cell -
totipotency.

The first mutants were obtained in the mid-70s using simple methods of breeding at callus tissues level.

Works in this area of research carried out in 80 years. List of mutants with important agricultural features is an extensive.

These include resistance mutants to stress factors, herbicides, various diseases, the top producers of essential amino acids. A powerful source of genetic diversity of plants is somaclonal variability that occurs during the passage of plants under uncontrolled growth, which allows for directional selection in vitro.

Despite the great promise of purposeful improvement of several traits of plants in vitro, cell breeding technology still remains though important, but only like a supplement to traditional methods of breeding.

Cell breeding examines the theoretical and practical aspects of the emergence of genotypic and phenotypic variability of cells and methods of breeding cells with new valuable properties. With modification of culture media can specifically affect to reproduction of cells with a given number of chromosomes in the nucleus. Theoretical basis of cell breeding is the provision of totipotency of cultivated plant cells. Transferred plant cell into culture conditions saves all the basic genetic information about the whole body and in appropriate conditions can implement it. Genetic changes, especially mutations that while there, after regeneration can occur in the form of new evidence produced in plants can enables by cell culture method to obtain new promising forms of breeding cultures.

Investigation of mutagenic effects of ionizing radiation on breeding purposes, which were started by Delaunay (1928-1930) and chemical mutagens (Rapoport 1943-1956) contributed to a trend in the field of experimental mutagenesis - mutation plant breeding. The successes achieved in vitro cultured plant cells made it possible to use them in breeding new plant forms.

The development of effective cell technologies acute following questions:
1. Obtaining a large number of cell cultures for plant breeding work;
2. Development of technologies directional influence on the genetic variability of cells that form these strains;
3. Development of technologies for a large number of viable fertile plants.

The main advantages of using cell cultures are:
- ability to manipulate millions of cells as mutagenic units;
- directional breeding in a Petry dish and regeneration of plants from mutant cells.

As an object for mutagenesis can be used:
- callus tissue;
- suspension culture;
- isolated protoplasts;
- sometimes pollen and seeds.

Selecting an object depends on the availability of the technologies developed for different species, and the ultimate goal of research.

Callus tissue is easily accessible material that is often used for the breeding of mutants sustainability. Applied a transplanted tissue that has not lost the ability to
regenerate. However, when dealing with callus cultures appear some drawbacks:
- Slow growth of callus tissues;
- Uneven for all cell effect of mutagens and toxic substances used as selective agents;
- Together with persistent possible survival of sensory cells that are in direct contact with the mutant. In this regard, it is possible allocation of chimeric cell lines.

Suspension culture. Get it from callus tissue by several passages in liquid medium. Used to produce cell lines resistant to antibiotics, amino acids and their analogs, salts of heavy metals.

Isolated protoplasts. Offers great opportunities for cell breeding of spontaneous and induced mutants. Bold of large homogeneous populations, haploid and diploid mesophilic protoplasts which actively divided it possible to study the quantitative mutagenesis of somatic cells and to analyze the expression of induced phenotypic changes at the cellular and organism level and their transmission in the offspring.

In the study of mutagenesis on cultured plant cells as a marker phenotypes using resistance to toxic concentrations of amino acids and their analogues, antibiotics, nucleic acid bases. The procedure for the breeding of mutants through the cell culture is characterized by specific phenotypic selection options at the cellular level, and by the regeneration of these plants and expression changes in the offspring.

For the breeding of different mutants using the following techniques:
1) Direct (positive) breeding, in which the survival of a particular search type mutant cells;
2) Indirect (negative) breeding, which is based on a breeding cell death that are divided unstable, but requires additional identifying mutational changes in them;
3) The total breeding, in which all individually tested cell clones formed;
4) Visual breeding and nonselective breeding when variant line can be identified among the entire population of cells visually or by using biochemical methods (thin layer or liquid chromatography, radio immune analysis).

Direct breeding is one of the most common method and used mainly for the breeding of mutants resistance to herbicides, antibiotics, toxins.

Scheme of direct breeding
- Treatment of cell by mutagens;
- Cultivation of cells in non-selective conditions (3-5 cell divisions);
- Cultivation of cells on selective media (selection of cell variants);
- Cultivation of colonies on regeneration medium (can recheck variant clones for resistance);
- Regeneration of plants and their reproduction (the tests for resistance, biochemical studies);
- Planting in soil (testing the stability of the seeds, the study of the genetic nature of resistance).

Indirect breeding method is effective for selection of conditional lethal mutants;
The total breeding is used to select defective as needed power mutants;
Visual breeding is used to select chlorophyll-defective mutants resistant to streptomycin and certain herbicides. For breeding of these mutants using the following concentrations of these substances in the environment, inducing greening does not inhibit tissue growth, and led to their discoloration. Mutants of stability has green color.

With selective breeding of new variants can be used biochemical approaches (thin-layer and liquid chromatography, radioimmunoassay analysis).

The process of cells cultivation and tissues in artificial media accompanied by various anomalies mitosis, leading to the occurrence of significant genetic diversity in populations of callus tissues cells.

**Different types of morphogenesis** - somatic embryogenesis and organogenesis - may also have different effects on genetic variation and consequently on plant phenotype. Phenotype and genetic changes are also among plants derived from isolated protoplasts. Variability of chromosome numbers of plants that were uncontrolled growth stage, in varying degrees, reflect this in cultured cells in vitro.

Subsequent studies have confirmed that the passage of cells under uncontrolled growth in vitro promotes the emergence of new forms of plants - somaclonal variants that differ from the original plant by phenotypic and genotypic characteristics.

The main reasons for somaclonal options are:
- genetic heterogeneity of somatic cell of original explants;
- genetic and epigenetic variability induced by the conditions of cultivation in vitro.

The frequency of formation somaclonal variants characterized by dependence on genotype and initial explants.

Many numerical cytological studies show that variability, induced by culturing conditions in vitro, is associated with genetic changes.

Regenerated plants derived from somatic cells and have some differences from the original forms called somaclones.

Nature and mechanisms of somaclonal variability:
1) natural genetic diversity of plant cells;
2) genome during cultivation in vitro, and that P.Larkin V.Skofkroft divided into the following categories:
   - Gross kariological change;
   - Cryptical invisible for cytological analysis chromosomal rearrangements;
   - The movement of mobile genetic elements;
   - Gene amplification and reduction;
   - Somatic crossing-over and exchange of sister chromatids;
   - Cryptical elimination of viruses.
3) cytoplazmon’s variability.

Somaclonal variability associated with hereditary reorganization of the genome marked newly discovered phenotypic changes that affect how epigenetic and can stably transferred daughter cells, but does not appear in regenerated plants and in their sexual progeny.

Promising research is somaclonal variability in the following areas:
1). Guided breeding of somaclones (depending on the needs of breeding work makes it possible to directed selection of options, but need to further explore the correlation
between resistance at the cellular level and the expression of these traits at plants;
2). Induced mutagenesis *in vitro* (can greatly expand the range of somaclonal variability and thus intensify research aimed breeding *in vitro*;
3). Transformation and transfer of specific genes (genetic engineering of plants by transferring individual cloned genes - the most effective way directional changes in the genotype of plants).

With the cell breeding were obtained plants resistant to drugs, amino acids and their analogues, herbicides stressors (salinity, heavy metals, drought, extreme temperatures, radiation stress), disease.

Getting of regeneration plants resistant to biotic and abiotic factors:
- Drought (polyethylene glycol, 99-880mM mannitol);
- Salinity (chloride and sulfate);
- Heavy metals (zinc, cadmium, copper, mercury, aluminum, cadmium);
- Extreme temperatures;
- Disease;
- Ionizing radiation.

**Resistance to drugs.** The underlying specific effect of antibiotics on plant cell, which is expressed in phenotypic discoloration photos synthesizing (green) or tissue inhibition of growth fabrics that are cultivated *in vitro*. Resistant lines selected on appropriate media for their ability to synthesize and grow under selective pressure of antibiotics.

**Resistance to amino acids and their analogues.** Cultivation of cells spend in media containing amino acids and their analogues. The mechanisms that cause resistance to amino acids and their analogues:
- reverse-weakening control of the biosynthesis of amino acids, which leads according to their supersynthesis. Supersynthesis of natural amino acid neutralizes the toxic effect of antimetabolites;
- the decrease in permeability analogue cells;
- collapse counterpart in the metabolism of non-toxic connection;
- violation of the features include analog in proteins.

**Resistance to herbicides.** Thanks to the inclusion in the culture media were obtained mutants herbicides resistance of carbamates to glyphosate, pikloramu, amitrolu, trazynu, sulfonylureas, roundup.

*In vivo* higher plants undergo the numerous stresses. Stress can be any external factor that causes a plant chemical and physical changes that affect the normal growth and development, leading to a decrease in the quality and stability of cultures. Particularly relevant is the research focused on the study of gene expression under external stress, stress-inducing proteins and the genes that encode them, which makes it possible to construct a plant resistant to environmental conditions.

For *in vitro* breeding of salt-tolerant plants using direct selection using callus or suspension cultures. In the same way selected lines resistant to heavy metals.

The stress caused by drought causes damage to plants caused by inactivation of enzymes violation of biochemical pathways, accumulation of toxic substances, lack of power. To work on cell plant breeding for drought tolerance thing is the presence of cellular mechanisms of osmoregulation. Such material is ethylene glycol field.
The cause of stress factors in plants may be relatively high and low temperatures. Cold stress can be caused by temperatures from 0 to +10-15°C. This stress plants suffer most tropical and subtropical areas.

Achievements and perspectives of cell breeding in the creation of new crop varieties.

The development of effective cell technologies an urgent question:
1) obtain for breeding purposes wheat and another agriculture cultures of a large number of cells strains of these plants;
2) development of technologies directional influence on the genetic variability of cells that form these strains;
3) technologies for reliable recovery of these large numbers of viable fertile plants;
4) development of methods for the breeding of plants with valuable somaclones genetically fixed characteristics and transfer them for further breeding work.

Cell technology in wheat breeding.
1) obtaining of callus culture of somatic cells and tissues using solid agar media.

THEME 5. ISOLATED PROTOPLAST CULTURE AND SOMATIC HYBRIDIZATION OF PLANTS

Plant cells surround by a dense and strong pectin-cellulose membrane. Neighboring cells of plant tissue have a common middle plate, which is surround on both sides by cellulose and hemi-cellulose microfibrils, connected by pectin compounds for durability. As a result, tissue cells firmly and tightly stitched together.

In the early 60-s of XX century English scientist Edward Kokinh proposed method of destruction of cell membranes, resulting in a living contents of the cell or cell membrane but not with plasma, cell membrane remains intact and viable. Cell which deprived mechanically or by enzymes of cell membrane, called protoplasts. This "naked" cells further potential to restore a new cell wall, divide and form cell aggregates, which can be obtained from plant regenerated.

Lack of cell wall allows to perform a series of genetic manipulations associated with the reconstruction of the genome, and get a population of hybrid cells after fusion of protoplasts isolated from the cells of origin or mutant cells of another species or even genus of plants.

Originally protoplasts were isolated by the method that included two phases of works. At the first stage, destroying pectin median plates to form a separate and interconnect cells. To do this, use enzymes – pectinase. In stage II single cells treated with cellulase enzymes to destroy the cell wall cellulose microfibrils.

Thus cells remained "naked" - without the cell membrane. Enzymes (pectinase and cellulase) are allocated by microorganisms that feed on plant debris. Microorganisms represent mold fungi - black and gray mold that often forms on plant debris and bread, as well as other species. During the cultivation of these microorganisms on nutrient media in the last stand enzymes that after a clean culture medium used for protoplast selection.

To isolate protoplasts, in addition to selected concentration, time of action of the enzymes used osmotic-substances to induce plasmolysis of the cell contents.
These substances are those that provide a high osmotic pressure in the medium for incubation: high concentration of sugar (>23%), solutions of sorbitol and mannitol (substances with high molecular weight) concentration 0.4-0.7 M. These osmotics dehydrated cells and separating it live content - protoplast - from cell membranes. Protoplasts collected in the form of balls inside the cell, protecting it from damage in the event of the destruction of the shell.

If the cell is elongated, protoplast can be separated into two parts in different parts of the cell: protoplast own with kernel and cytoplasm. After the destruction of the cell membrane of isolated protoplasts necessarily washed free of enzymes and sow them in culture medium with the presence of mannitol or sorbitol to maintain a high osmotic pressure. It is necessary to thin cytoplasmic lipid-protein protoplast membrane is not torn as in protoplasts retained a high osmotic pressure.

Found that washed isolated protoplasts from enzymes able to restore cell membrane to the culture medium is again converted into cells. This process begins immediately after washing enzymes and cells resemble typical callus cells than the original cell explants. They divide rapidly, forming callus from which later formed buds, stems, shoots, roots (Pic. 12).

Before the formation of cell membranes protoplasts, surrounded by a thin outer membrane can associate (merging) to form a single cell with a few even taxonomically distant species. Protoplasts can absorb from the environment not only low-molecular substances (ions, sugars, amino acids) and macromolecules. This absorption of large molecules associated with a particular form of transport agents directed inwards. It enables to change the genetic properties of the cells (there are new features). Genetically modified cells initiate new properties for unusual plants that can not the classic methods of genetics.

From a practical point of view it is interesting construction plant cells using isolated protoplasts with different genetic characteristics as the nucleus, plastids and other cellular organelles of different origins belonging to different species. The merger of two protoplasts - one of the core, more - only with the cytoplasm, formed cybrids.

1. Obtaining protoplasts.
   There are two ways of destruction of cell membranes - mechanical and enzymatic. Mechanical method is limited to low protoplast yield and narrow range of
species to which it is applicable. The first report on the use of enzymes for the release of protoplasts was in 1960.

Protoplasts from roots cells of tomato were obtained by processing of crude cellulase enzyme obtained from the fungus *Myrathecium verrucaria*. Moreover, significant progress toward technology selection protoplasts associated with industrial production of enzymes that destroy cell membranes.

By the most commonly used enzymes for the release of protoplasts are drayselase of *Basidiomycetes* (enriched of cellulase and pectinase), pectinase *Rhizopus* etc.

For obtaining protoplasts using one-or two-phase processing of materials by enzymes. For two-phase method plant material initially treated with pectinase to select cells from tissues. The cells were then filtered, centrifuged, removing remnants of tissue and a solution of pectinase, destroying cell membranes cellulase preparation, thus releasing protoplasts. Often, to obtain protoplasts from tissues of different species using single-phase method with a mixture of processing of enzymes cellulase and pectinase. For optimum output of viable protoplasts in the grass using complex mixture of enzymes, including pectinase, cellulase, and drayselase, rozym.

Enzyme mixture to isolate protoplasts prepared in osmotic stabilizer. Recommend using metabolically active stabilizers (glucose, sucrose, sorbitol) with metabolically inert (mannitol), so that the first active protoplasts absorbed during their growth and formation of cell membranes, which leads to a reduction of the osmotic pressure in the medium for culturing protoplasts.

**The starting material for the release of protoplasts**

Protoplasts isolated *in vitro* cultured cells and tissues, but the most commonly used sources for these purposes is the leaf tissue and cell suspensions (Pic. 13).
Before the release of protoplast surface sterilized leaves, lower surface of leaf epidermis is removed or carborundum rub, or to improve the penetration of enzymes leaves densely notched. Preferably use sterile leaves of plants cultured in vitro, because it provides uniformity original tissue, the presence of sterile material in any season of the year, regardless of season and high repeatability of results. The action of enzymes from damaged cell membranes increased use of vacuum processing and mixing mortar on rocking.

Number of protoplasts and their stability during isolation depend on such factors as a means of sterilization of the source material, the type of enzyme, its concentration, vacuum infiltration of treated enzyme material volume incubation solution stirring during isolation, temperature, concentration of the osmotic solution, the addition of divalent cations and polyamines to enzyme mixtures.

**Cleaning protoplasts**

After the destruction of cell walls purified from protoplast suspension cells and tissue debris by filtration through a mesh or filters with a diameter holes from 40 to 150 microns (Pic. 13). Enzyme reaction mixture is removed by centrifugation, followed by 3-4x resuspension in wash solution or culture medium. Sometimes use washing protoplasts by flotation in concentrated solutions of sucrose, mannitol or fikolu. To effectively remove cell debris, and especially cell organelles that pollute protoplast culture, effective density gradient centrifugation to separate and the two-phase method.

*Pic. 13. Scheme of receiving and cultivating protoplasts (Atanasov, 1988)*
2. Cultivation of proplasts.

After cleaning the proplasts resuspended in culture medium. The minimum density in this case is 104 in 1 ml of culture medium. However, a suitable density of proplasts is 105 in 1 ml.

There are different methods of culturing proplasts:

Method microdrops. Used for the cultivation of a small number of proplasts, and if testing culture media in research to optimize cultivation conditions. Redwood drops less than 50 ml. To maintain proper humidity in the chamber in the middle of the Petri dish is placed a few drops of sterile water.

Proplast suspensions. Proplasts suspended in a thin layer (1 mm) of liquid medium in Erlenmeyyer flasks with a volume of 25 ml or in Petri dishes (diameter 60 mm) with or without shaking.

Playing method. Proplasts, suspended in a liquid culture medium, mixed with the same volume of culture medium with agar for use in a water bath maintained at 45 °C. For optimal aeration is necessary to blend bottom cups covered with a thin layer.

Proplasts can be suspended in semisolid medium (0.4% agar) or on the surface of the culture medium (0.8% agar). In some cases, the best form semisolid medium colony. A larger divisions of proplasts when applying them to the surface of filter paper that is on the agar medium.

Cameras for microcultivation of proplasts produced in this way: on a glass slide is put a drop of the culture medium (30 ml) containing one or more proplasts. On both sides of the drop is placed for cover glasses, which put another piece of glass coverslip. To prevent drying, microcamera isolated sterile paraffin. Recently, the use of small Cuprak cups numbered slots into put a drop of culture medium with volume 0,25-25 ml, creating a plurality of rows of droplets.

Cultivation on - "nutrient layer." Indivisible, but with an active metabolic cells irradiated with X-ray or gamma-rays and immersed in the culture medium can support the growth of proplasts even at very low density latter (5-50 proplasts at 1 mm). Co-cultivation of "nutrient culture" is used in the cloning of somatic hybrids.

Cultivation of mobilized proplasts. Proplasts, coated with calcium alginate, able to keep the pressure in the osmotic potential of the culture medium, which can be used in their cultivation. Found that the decrease in the osmotic pressure of the culture medium increases the viability of proplasts of some species compared to controls.

3. Regeneration of plants from proplasts

In the process of culturing viable proplasts regenerated cell membrane and become normal cells cultivated in vitro. Predecessors of cellulose and pectin are synthesized mainly in the Golgi apparatus, but can be formed in other cellular structures, such as the endoplasmic grid controlled by the kernel. Proplasts with nuclei (cytoplasts) can not restore a new cell membrane.

The formation of the cell wall of proplasts begins immediately after washing enzymes with which they were received. After 24-36 h observed first cell division, and 3-4 weeks callus cells formed colonies. During cultivation gradually (over a week) reduces the osmotic pressure in the culture medium to accelerate cell division.
Then callus cultivate on agar medium for plant regeneration (Pic. 14).


**Fusion of protoplasts**

During protoplast fusion takes place first of agglutination (clumping), then proper membrane fusion. After removal of the cell wall of plasmodesmata increase in size by combining the contents of two or more cells into one. Protoplasts have a negative surface charge as mutually repel. For a merger of this repulsion is overcome and removal or redistribution of the surface charge. For induced protoplast fusion using chemical and electrical methods.

The chemical method is added to a suspension of protoplasts substances that stimulate fusion (nitrates). To neutralize the negative charge on the surface of protoplasts used as Ca$^{+2}$ (50 mM) at pH 10.5.

Through search effective fuzogene (inductor merger) was found polyethylene glycol (PEG) - soluble polymer in water. Pretreatment of mixed protoplast suspension concentrated solution of PEG (20-30%) is their adhesion. After 10-15 minutes PEG removes by solution with an alkaline pH (9-11) and high Ca$^{2+}$ (100-300 mM), and this solution protoplast membrane clusters merge.

Using PEG can be drained from 10 to 50% of the protoplasts. There are various theories to explain the mechanism of protoplast fusion under the influence of PEG: due to dehydration, glycol absorbs free water between protoplasts, breaking the double layer membrane and facilitating the fusion of the membrane exposed places, PEG reduces the polarity of the water environment, which redistributes polar and hydrophobic components of membranes, stabilizing lipid structure of the membrane, induces the formation of PEG pores on the entire surface of the membrane, through which flow occurs intracellular material immediately after the merger section of the
membrane with pores for a while stored. Fused protoplasts rounded and subject to repeated injuries occur regeneration of cell walls and cell division formed.

Methods of cell engineering, which refers primarily protoplast fusion, creating a new type of cell and based not only on the hybridization of whole cells, but also by reconstructing cells from individual fragments of different cells. Genetic reconstruction of plant cell input it organell isolated cell (nucleus, chloroplasts and mitochondria) allows purposefully transmit hereditary characteristics that are encoded cytoplasm, create new forms of economically important crops. Thus, the use of highly efficient chloroplast can activate photosynthesis, contributing to increased productivity of the plant. Chloroplasts codes for herbicide tolerance, immunity to certain diseases, reactions to toxins. Merging protoplasts with mitochondria that control cytoplasmic male sterility (CMS), can wish to plant this valuable feature.

![Diagram](image1)

**Pic. 15. Scheme of merging protoplasts with PEG:**
A – complex of protoplasts; B – merging protoplasts in the result of degidratation with PEG; C – pass of inside cell composition in consequence of creation pores on membranes; D – merging products with pores on membranes; E – hybrids and cybrids products of merging (Bornman, 1991).

![Diagram](image2)

**Pic. 16. Stages of merging protoplasts under action of electric field (Bornman, 1991)**

**Methods for breeding of hybrid cells and plants**

The merger protoplasts has two types of hybrid cells - homocariones (composed of cells of one parent) and heterocariones (composed of cells of both parents).

Successfully obtaining somatic hybrids is largely dependent upon the
effectiveness of the identification and selection of fusion products. Selection can be made immediately after the merger or procedures at various stages of cultivation of somatic hybrids.

**Genetic complementation**

To prove the hybrid nature of the regenerated plants from the hybrid cells using genetic complementation. Genetic complementation - it is the interaction of genes in hybrid cells, which resulted in reduced function of the defective gene.

Mutations controlled by chloroplast genes characteristic of many cultivated plants and the use of genetic complementation based on them is one of the most reliable and easiest ways to identify somatic hybrids.

Auchsotrophes - a biochemical mutant cells due to mutation lost the ability to grow in normal culture medium and in need of additional substances, the synthesis of which they blocked mutation.

Pic. 17. Experiment of merging haploid protoplasts both varieties (Valihanova, 1996)
Using physiological complementation initially studied cultivation conditions for the growth of the parent form and find suitable for the growth of cells of a father and unsuitable for another. Then the cell suspension was placed in series after the merger of these different culture media in each of these cells are killed by a parent, but continue to grow and develop hybrid cells.

**Using of biochemical mutants**

For the breeding of somatic hybrids efficient using a series of dominant and recessive biochemical mutants. These include a series of mutants of tobacco with nitrotreduktase failure, aucsotrophes mutants, dominant carrot mutant and tobacco with resistance to amino acid analogs. Typically derived somatic hybrids have a double resistance, manifested in their cultivation on nutrient media suitable composition. One of the most effective markers in the breeding of somatic hybrids are resistant to antibiotics - kanamycin, streptomycin, hygromycin, etc. and herbicides - glyphosate, phosphynotrucyn etc.

These biochemical markers obtained by transformation with *Agrobacterium tumefaciens*, using the genetic structures that carry genes synthesizing hormones *Agrobacterium*, and "genes reporters" - GUS (glucuronidase) and luciferase. Marker system has been successfully used in experiments with somatic hybridization in several species, as well as in the preparation of intra-and interspecific hybrids of tobacco.

**Breeding for growth intensity.** An additional marker for somatic hybridization may serve heterosis effect against the growth of callus such an effect is observed in all interspecific hybrids genera *Datura, N. glauca + N. langs-dorfii, N. glauca + N tabacum, Datura inoxia + Atropa belladonna, Solanum tuberosum + S. chacoense and Medicago sativa + M. falcata*.

**Breeding based on morphological characteristics.** Some hybrids of different plant species identified by the morphology of the flowers and leaves. Breeding of hybrid combinations at the level of whole plants more efficient than breeding at the cellular level, so that it acts long after the merger. However, while a number regeneration could be identified at the level of the hybrid cells are lost.

To identify somatic hybrids sometimes used iso-osmotical various of density gradient. The protoplasts mixture centrifuged in sucrose density gradient, which allows to select many heterocariones, but their share is never 100%.

**6. Analysis of somatic hybrids**

To confirm the hybrid nature derived from fusion of cell aggregates plant-regenerants need of cytological, biochemical and molecular genetic analyzes. Some of these methods are used at the stage of breeding of hybrid cells and plants.

**Cytological analysis**

Hybrids are often analyzed by cytogenetic methods that examine the number and morphology of chromosomes. For species in which chromosomes are morphologically indistinguishable, using the method of differential staining, which detects specific structure of each pair of homologous chromosomes. Cytogenetic analysis is not always reliable due to the high variability of chromosome cells *in vitro*.

Merge of cells which divide, nuclei are in interphase stage, usually leads to
increased mitotic activity past or premature chromosome condensation. It is believed that most heterocariones nuclear cycles (though not all) are synchronized, the chromosomes are oriented in the common metaphase plate and the end of the first mitosis are surrounded by a common nuclear membrane. Often there are irregularities in the arrangement of chromosomes, as described, for example, the hybrid *Nicotiana chinensis + Atropa belladonna*.

Note that the somatic hybrids characteristic cytological instability. For example, changing the number of chromosomes (poly- and aneuploidy) and chromosomal rearrangements occur at the level of cell colonies hybrid between *Vicia hajastana + Glycine max, V.faba + Petunia hybrida, Nicotiana glauca + G. max, Arabidopsis + Brassica, and at the level of entire hybrid plants in tobacco, petunia, carrot, dope, intergeneric hybrids *Atropa families and Datura, Arabidopsis and Brassica, Lycopersicon and Solanum, N. chinensis i Atropa belladonna.*

The causes of genetic instability of somatic hybrids are not completely understood. On the one hand, they may be the result of phylogenetic distance of the source species, on the other - this process may include other factors, as in some interspecific somatic hybrids observed cytological deviation greater than intergeneric hybrids. Duration of callus passage induction and somatic hybrids also affects the cytological instability. In particular, the observed chromosome rearrangements, activation-band transport vessel elements, gene amplification, somatic crossing over and exchange of sister chromatids. Thus, the earlier regeneration occurs, the less can be cytologically changes. In cytological stability of somatic hybrids affects the composition of culture media.

**Biochemical analysis**

Biochemical methods are widely used for the analysis of interspecific hybrids. Study by polyacrylamide gel electrophoresis followed by staining proteins with defined enzymatic activity is the easiest and most effective means of biochemical analysis of paresexual hybrids. To this end, studying the spectra of peroxidase isozymes, esterase, malate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate, amylase and other enzymes. One of the most reliable and most affordable methods of biochemical analysis of somatic hybrids is RubisKO. This is the most common and most studied plant protein, a key enzyme of Calvin cycle and consists of large and small subunits. Large polypeptides subunit encoded chloroplast DNA of small-nuclear DNA.

Study of polypeptide subunits by polyacrylamide gel allows to detect differences in the structure of polypeptides of different protein species. Such studies conducted in interspecific somatic hybrids in order to establish their hybrid nature. Analysis RubisKO also successfully used in the case study under nuclei genes during paresexual hybridization. The hybrid nature of somatic hybrids can be confirmed by the presence of isozyme bands characteristic of both parents. Isozyme spectra may be submitted in its entirety or individual bands may be absent or may be new, not characteristic of the original parental plants. Of course, these deviations are the result of cytological or genetic instability in somatic hybrids. RubisKO - one of the key enzymes of photosynthesis, which consists of two polypeptide subunits. Peptides encoded small subunit genes of the nucleus, and large - plastid genes.
This property is used as a means to confirm the hybrid nature derived somatic clones. In order to establish the truth of hybrids are researched and chloroplast DNA and mitochondrial DNA hybrids and their parents. These nucleases cleave DNA at various specific locations. For the analysis of somatic hybrids used as the method of molecular hybridization of nucleic acids - as DNA-DNA and DNA-RNA. Molecular hybridization is an important criterion in the case study of hybrids phylogenetically distant species and asymmetric hybrids.

These methods have proof that obtained after protoplast fusion cells (or plants) are not chimeras or mutants that phenotypically replicate hybrid.

**Molecular and genetic analysis**

Use of DNA restriction analysis for the study of chloroplast and mitochondria of somatic hybrids has some advantages:
- broad scope of application of the method, since the restriction sets differences can be identified more frequently than during electrofocuse;
- increase the resolution compared to the above methods;
- the ability to detect DNA restructuring, whole genome.

Promising method may be the use of ribonucleic acid, repetitive DNA sequences as probes for DNA restriction fragment core as well as the method of hybridization *in situ*. Content and spectra of some secondary compounds such as alkaloids, may also be useful to determine the hybrid nature of the merging when creating somatic hybrids.

### THEME 6. GENETIC ENGINEERING OF PLANTS AND THE INTRODUCTION OF ITS ACHIEVEMENTS IN THE PRODUCTION

Contents:
1. Molecular basis of heredity;
2. DNA technology in genetic engineering;
3. Isolation and gene transfer;

1. Genetic Engineering - is the newest trend in genetics and biotechnology, whose main objective is purposeful alteration of the genome of organisms through changes in their genetic information using artificial methods of gene transfer. Among them the most important are:
   - Synthesis of genes outside the body;
   - Discharge from the cells of individual genes or genetic structures;
   - Directed the restructuring of selected genetic structures;
   - Copying and reproduction of isolated or synthesized genes and genetic structures;
   - Communities in a different cell genomes (construction of a new genotype).

   Genetic engineering is a radical new technology, one that breaks down fundamental genetic barriers, not only between species, but between humans, animals, and plants. By combining the genes of dissimilar and unrelated species, permanently altering their genetic codes, tale organisms are created. GE places in human hands the capacity to redesign living organisms, the products of some three
billion years of evolution.

The main objective of genetic engineering of plants is enriching varieties with new features: resistance to herbicides, pests, pathogens, best quality composition, improved taste and nutritional properties.

In 2002, the global area under transgenic crops were 58.7 million hectares, which is almost twice the territory of England. Compared with 1996, the global area under transgenic crops increased 35 times. The main area under transgenic crops (99%) belong to four countries: two highly - USA and Canada and the fact that developing countries - Argentina and China.

Chromosomes are the storage place for all genetic, that is hereditary and transmissible information. This information is written along a thin thread (string) called DNA. 'DNA' is an abbreviation for deoxyribonucleic acid, a specific acidic material that can be found in the nucleus (center). The genetic information is written in the form of a code. To ensure the thread and also stable (secure) and safe information, a twisted double thread is used, the famous double helix. When a cell multiplies it will also copy all the DNA and pass it on to the daughter cell.

The totality of the genetic information of an organism is called genome. Cells of humans, for example, possess two sets of 23 different chromosomes, one set from the mother and the other from - the father. The information contained on the chromosomes in the DNA is written and coded in such a way that it can be understood by nearly all living species on earth. It is thus (accordingly) termed (named) the “universal code of life”. In this coding system, cells need only four symbols, called nucleotides, to spell out all the instructions of how to make any protein.

Although it is true that genes are specific sequences of DNA that are central to the production of proteins, contrary to popular belief and the now outmoded (out of date) standard genetic model, genes do not directly determine (control) the traits (qualities) of an organism. They are a single factor among many. They provide the 'list of ingredients' which is then organized by the 'dynamical system' of the organism. That 'dynamical system' determines how the organism is going to develop. In other words, a single gene does not, in most cases, exclusively determine either a single feature of our bodies or a single aspect of our behavior. No gene ever works in isolation, but rather in an extremely complicated genetic network. The function of each gene is dependent on the context of all the other genes in the genome.

One of the key techniques in genetic engineering is a recombinant DNA techniques, or so-called DNA technology. They are based on the ability of specific enzymes recognize specific sites in DNA molecules - restriction, leading to the formation of gaps at certain points and "stitching" fragments obtained with other DNA molecules by other enzymes - ligase. These fragments can be replicated (cloned) in large quantities in special vectors (plasmids of bacteria and bacteriophage DNA).

Issues that are resolved by using genetic engineering techniques, the following: that the structure and function of genes, how many and where they are, which ones can be isolated and cloned, which are marker genes can be used to transform, what are the mechanisms of plant gene transfer, as regenerating changing ability of
transformed plant cells and what it depends, what regulatory mechanisms introduced DNA and others.

Genetic information is stored in nucleic acids transferred to them by inheritance. In all cellular organisms and viruses containing DNA information transfer by molecules of DNA, and RNA-containing viruses - RNA molecules.

As a carrier of genetic information, DNA, has two main functions:
- Self reproduced during replication (doubling) before cell division so that each daughter cell has the same information;
- Sends information encoded therein information of RNA molecules in the process of transcription, in turn, mRNA conveys information recorded 4-letter alphabet of nucleic acids (A, T, G, C), by broadcasting a 20-letter alphabet of proteins (alanine, asparagine, valine, glycine, glutamine ...)

Specific groups of nucleotides in the mRNA molecules that correspond to individual amino acids, called codons. The basic properties of the genetic code:
- triplet code, each amino acid is encoded by three nucleotides;
- one and the same amino acid can be encoded by not one but several nucleotide triplets;
- nitrogenous bases codon for one amino acid is not part of the neighboring amino acids;
- the genetic code is universal, that is, for any kind of body of each of the amino acids encoded by the same codon.

2. The main objective of DNA technology - a selection of individual genes and their molecular cloning (propagation), creating recombinant DNA - an artificial combination of genes and promoters.

Isolation and cloning of genes by using three types of enzymes:
- restricts;
- ligase;
- reverse transcriptase.

Restricts - bacterial enzymes that cut DNA at short or long intervals, the individual nucleotides. They feature is the specificity - each cut DNA in a strictly defined place between individual nucleotides. There are more than 100 restriction.

Ligases - enzymes that are "stitched" free ends of DNA through the formation of a single polynucleotide.

Reverse transcriptase (revertases) - enzymes, like DNA polymerase, but not to synthesize DNA chain DNA and RNA (which is typical of RNA-containing viruses).

Cloned genes transferred into a cell (transform it) via vectors using plasmid bacteria and bacteriophages and viruses.

Plasmid (episoma) - without-chromosome genetic element, which is a circular double-stranded DNA molecule. It prevents the cell from entering other plasmids of the same type using the principle of incompatibility.

Of course plasmid restriction digestion cut in one place, there is introduced genes required, add ligase, which again closes the ring structure of DNA plasm included alien gene. Then plasm by "contamination" is transferred into any cell, where he began to work the new genes introduced in the plasm. Methods of experimental interventions that allow to continue as planned restructure the genome.
of organisms, altering its genetic information as the basis of genetic (genetic) engineering.

Classical processes (methods) genetic engineering are:
- Synthesis of genes outside the body;
- Discharge from the cells of individual genes, fragments of chromosomes, entire chromosomes, nuclei and other organelles;
- Targeted alteration isolated structures;
- Copying and reproduction of isolated or synthesized genes or genetic structures;
- Transfer and introduction of genes or genetic structures in the genome you want to change (transhenoz);

3. Isolation and gene transfer. Moving isolated from the genome or artificially synthesized genes in another genome is often measured using a method involving the use of segments of DNA with "sticky" ends - segments that ends with such a sequence of nucleotides that can form a complementary pair with the DNA segment to which they want to connect. For the transfer of genetic information using vectors.

Vector - a specific structure which is able to replicate and which can attach to their genes and transfer them to other cells, a nucleotide sequence capable incorporated into DNA (without violating its integrity). Vectors are called modified plasmid, bacteriophage, virus, yeast or bacterial DNA or RNA, to ensure penetration of exogenous DNA into the host cell. Today vectors are designed to transfer (transformation) of foreign genes in cells of higher plants. There are the following groups:
- The vectors derived from bacterial plasmids (Ti-plasmid agro bacteria and Ri-plasmids);
- Vectors are designed based on the DNA and viral pathogens plants;
- The vectors that may exist in plant cells as independent replicon (mitochondrial and chloroplast DNA).

Most promising vectors constructed from plasmid Ti and Ri. The relationship between soil bacterium Ti-plasmid and dicotyledonous plants causing crown formation cancer and bacteria Ri-plasmids - the formation of "bearded roots." Then plant cells cultivated in the medium with the addition of hormones, and after 3-4 weeks, small colonies planted on no hormonal environment. In this environment only surviving colonies of transformed cells. Thus-obtained transformed plants regenerated tobacco and petunia, cereal plants.

Direct gene transfer is achieved:
- mechanical means;
- the action of certain chemicals (PEG, polyvinyl alcohol, etc..)
- electroporation (by an electric current), which lies at the heart of punching electrocution cell membranes.
- microinjection by microdrops into mesophillous leaf cells in a small amount of solution injected DNA (nucleus or cytoplasm, after which survives 50-90% of cells, many of which regenerates the trans gene plants;
- microbombing - bombing micro particles, coated DNA of plant cells. In this system micro particles of tungsten or gold coated with DNA are accelerated by
compressed helium or electrical discharge through the device, known as a "gene gun". The method is called ballistic transformation or biolistics.

Retrieved many species of plants transformed by using microbombing: wheat, corn, rice, rye, oats, soybeans, sugar cane and others.

Transfer and inheritance of foreign genes would have been impossible without the appropriate promoters and terminators.

Promoter - a stretch of DNA that is located in front of the site of transcription of the gene, which binds RNA polymerase.

Terminator - is a specific piece of DNA that is at the end of the gene responsible for stopping the synthesis of mRNA. Mainly depends on the properties of promoter ability of RNA polymerase to initiate transcription.

4. Scale advances in genetic engineering of plants currently modest compared to other biotech areas (microclonal reproduction, somatic hybridization, etc.). But genetic engineering opens up new prospects for plant breeding related to the ability to transfer genes from bacteria, fungi, exotic plants and even humans and animals, and thus opens up possibilities beyond the reach of experimental methods of mutagenesis and conventional breeding.

Recently, there is a need to create more productive forms of plants that could not only combine qualitative and quantitative traits, but also resistance to biotic and abiotic environmental factors.

Transgenic soybean and corn plants with improved qualities of fat, very low in polyunsaturated fatty acids and high levels of monounsaturated acids. Some of these modified fats are used in industry as lubricants. Cloned genes are storage proteins of soybeans, peas, beans, corn, potatoes and many others. One way to create a more complete protein in legumes is to use gene 2S-protein of nut protein that misty plenty of methionine. Thus the obtained transgenic form bean. Creation of transgenic clover plants containing a gene fusion protein sunflower with a high content of sulfur-containing amino acids.

Genetic engineering is used to take genes and segments of DNA from one species, e.g. fish, and put them into another species, e.g. tomato. To do so, GE provides a set of techniques to cut DNA either randomly or at a number of specific sites. Once isolated one can study the different segments of DNA, multiply them up and splice them (stick them) next to any other DNA of another cell or organism. GE makes it possible to break through the species barrier and to shuffle (mix) information between completely unrelated species.

In order to avoid long testing and adjusting (regulating), most genetic engineering of plants is done with viral promoters. Viruses are very active. Nothing, or almost nothing, will stop them once they have found a new victim or a host. They integrate their genetic information into the DNA of a host cell (such as one of your own), multiply, infect the next cells and multiply. This is possible because viruses have evolved very powerful promoters which command the host cell to read the viral genes constantly and produce viral proteins.

There are different ways to get a gene from A to B or to transform a plant with a 'new' gene. A vector is something that can carry the gene into the host, or rather into the nucleus of a host cell. Vectors are commonly bacterial plasmids or viruses.
Another method is the 'shotgun-technique', also known as 'bio-ballistics,' which blindly shoots masses of tiny gold particles coated with the gene into a plate (cover) of plant cells, hoping to land a hit somewhere in the cell's DNA.

Often genetic engineering will not only use the information of one gene and put it behind the promoter of another gene, but will also take bits and pieces from other genes and other species. Although this is aimed to benefit the expression and function of the 'new' gene it also causes more interference and enhances the risks of unpredictable effects.

Bioengineers often claim that they are just speeding up the processes of natural selection and making the old practices of breeding more efficient. In some cases that may be true, but in most instances the gene changes that are engineered would never occur in nature, because they cross natural species barriers.

Furthermore we can say that GE is a test tube science and is too early applied in food production. A gene studied in a test tube can only tell what this gene does and how it behaves in that particular test tube. It cannot tell us what its role and behaviour are in the organism it came from or what it might do if we place it into a completely different species. What seems to be the case in the laboratory may or may not be valid in the natural world. Therefore, we cannot know through scientific method the full extent (amount) of the possible effects of genetic alterations in living creatures. For example: genes for the colour red placed into petunia flowers not only changed the colour of the offspring but also decreased fertility and altered the growth of the roots and leaves. Salmon genetically engineered with a growth hormone gene not only grew too big and too fast but also turned green. These are unpredictable side effects, scientifically termed pleiotropic effects.

How do we know that a genetically engineered food plant will not produce new toxins and allergenic substances. How about the nutritional value? And what are the effects on the environment and on wild life? All these questions are important questions and yet they remain unanswered. Until we have an answer to all of these, genetic engineering should be kept to the test tubes. Biotechnology married to corporations tends to ignore the precautionary principle and basic scientific principles.

Nowadays many companies try to alter plant’s genes in that way, so that they are more resistant to herbicides, which are often sold by the same company.

For example: One of these companies is Monsanto. Monsanto's new genetically engineered soybean has been modified so that it can survive heavy doses of Monsanto’s poisonous weed-killing herbicide Roundup. Agricultural business already dumps (leave) more than 500 million pounds of herbicides on U.S. farmland each year, with Roundup leading the toxic parade. Herbicides contaminate ground water and the food chain, contributing to the cancer epidemic which now strikes one in three citizens. A study released in August 1995 found that levels of herbicides in drinking water exceed (go beyond) federal safety levels in 29 towns tested in the Corn Belt.

Monsanto’s soybeans, called 'Roundup Ready', have been approved for commercialization this year. Approvals for other such products are imminent.

Another example: Calgene's new transgenic canola (rapeseed) plant, spliced
with genetic material from the California bay plant, can produce lauric acid, a
substance used by industry to produce soaps, chocolate and other foods. It’s
commercialization should be opposed because the plant will wreck the Third World-
based coconut and palm kernel (core) oil industry which currently exports hundreds
of millions of dollars-worth of lauric oils to the U.S. each year. The product, called
'Laurical', has been approved for commercialization this year.

On March 3, 1998 the US Department of Agriculture (USDA) and an
American cotton seed company, Delta & Pine Land Co., received a US patent on a
technique that genetically alters seed so that it will not evolve if re-planting a second
time. The technology aims to prevent farmers from saving seed from their harvest
(crop) to re-plant the following season. Because it is a potentially 'deadly' technology,
Rural Advancement Foundation International (RAFI) has named it the 'Terminator
technology.' If commercially possible, the Terminator technology will have deep
implications for agriculture. It is a global threat to farmers, biodiversity and food
security. The seed-sterilizing technology threatens to eliminate the age-old right of
farmers to save seed from their harvest and it endangers food security in common.
Delta & Pine Land Co. and USDA have applied for patents on the Terminator
technology in at least 78 countries. If the Terminator technology is widely used, it
will give the multinational seed and agrochemical industry an extraordinary and
extremely dangerous capacity to control the world's food supplies.

Therefore the patenting of genetically engineered foods and widespread
biotech food production by major international chemical, pharmaceutical and
agricultural corporations will eliminate farming, as it has been practiced since the
beginning of human’s appearance on the planet, if the trend is not stopped.

We should also consider the fact that many scientists have claimed that the
ingestion of genetically engineered food is harmless because the genetically
engineered materials are destroyed by stomach acids. But recent research suggests
that genetically engineered materials are not completely destroyed by stomach acids
and that significant portions reach the bloodstream and also the brain-cells.
Furthermore, it has been shown that the natural defense mechanisms of body cells are
not entirely effective in keeping the genetically engineered substances out of the
cells.

Some dangers of eating genetically engineered foods are already documented.
Risks to human health include the probable increase in the level of toxins in foods
and in the number of disease-causing organisms that are resistant to antibiotics.

The major risks of eating genetically engineered food are:

The new proteins produced in genetically engineered foods could: a) themselves, act as allergens or toxins, b) alter the metabolism (Stoffwechsel) of the
food producing organism, causing it to produce new allergens or toxins, or c) causing
it to be reduced in nutritional value.
THEME 7. CRYOPRESERVATION METHODS AND PLANT CELLS 
BANKS - THE BASIS OF NEW BIOTECHNOLOGIES

Contents:
1. Cryopreservation methods;
2. Tests to determine cell viability;
3. Banks of genetic resources.

1. The possibility of increasing yields and improving economically valuable 
traits of plants provide the presence and support of various collections and 
germplasm bank of plant genetic resources (genetic banks). This may include 
different forms of plants (relic, endemic), and forms created by traditional methods or 
with the involvement of non-traditional methods - tissue culture, cell and genetic 
engineering.

An effective way to preserve the gene pool of plants is conservation seed or 
plant parts in freezing conditions. For seeds is sufficient temperature - 18-20 ° C and 
humidity of 3-7%, in which storage can be carried out for 50-100 years. The most 
promising way to long-term storage and cryopreservation of seeds is storing it in a 
pair of liquid nitrogen (- 160 ° C) or liquid nitrogen (- 196 ° C). In genetic banks of 
some countries (Poland, China, Japan, USA) reliable means of keeping the gene pool 
of plants is cryopreservation - keeping the temperature of liquid nitrogen - 196 ° C. 
Under these conditions it is possible to suspend those genetic changes that occur 
during long-term cultivation of cells and tissues in vitro.

So currently the only way for unlimited storage of strains of cells, tissues, 
organs of plants is cryopreservation. These terms are complex multistage process that 
is used for long-term storage in intact living cells, tissues and organs in a state of 
suspended animation.

Milestones scheme of cryopreservation:
• aseptic isolation and cultivation of plant tissue;
• adaptation to low temperatures (if necessary);
• preliminary culturing material in an appropriate culture medium supplemented by 
cryoprotectors for a certain period;
• adding cryoprotectors to samples at the preparatory stage of freezing;
• check cooling and freezing of samples;
• storage in liquid nitrogen;
• fast thawing of plant tissues;
• reuse of recovered tissue culturing and induction of regeneration.

Cryopreservation methods:

1) Freezing buds and meristems. Cryopreservation genetic resources meristem is 
superior to other methods. Because the meristem develop directly into plants while 
ensuring high genetic stability, relatively easily withstand freezing as consisting of 
small cells, which do not have vacuoles. To freeze meristems often use immersion in 
liquid nitrogen or freezing of gradual cooling. The results of the meristem peas and 
strawberries show that when the cooling rate is 0,6-0,8 ° C for 1 min, kept high 
enough vitality - 95%, and 26 after 8 weeks of storage in liquid nitrogen, it remains at
2) **Features of the freezing of cell cultures and tissues.** Appropriate samples of cell cultures are placed in special vials 2 ml under sterile conditions. In the bottom usually hold 1 million cells. First, conduct a slow cooling at the same rate, then gradual freezing under the influence of low temperatures single or multiple, then use immersion in liquid nitrogen at a temperature of -196 °C. Defrosting is carried out for 1-2 min, provided that the tubes swaying.

3) **Freezing of protoplasts.** Protoplasts differ significantly from cell suspension: they have no cell wall and cytoplasmic contact with neighboring cells. Use a mixture of 5% dimethyl sulfoxide (DMSO) and 10% glucose. Other procedures are not in principle different from the same for cell culture.

2. To determine the viability of cell cultures using the following tests:
   - Painting flyuorescyndiatsetate (FDA), only living cells can colored by FDA and fluoresce under ultraviolet light.
   - 2,3,5-threephenyltetrazoliumchlor method (TTC). Cell survival after turning into formazon that there is a red substance, insoluble in water (soluble in the case of addition of ethanol).

**Factors affecting the viability of the cells after cryopreservation:**

1. **The type and physiological state of cultures before freezing.** For example isolated meristem plants that naturally resistant to low temperatures, characterized by a high viability after thawing than, for example rain. Cells after mitotic division resistant to freezing than cells that are in different phases of the mitotic cycle.

2. **Type of cryoprotectants.** The degree of survival of plants after storage in liquid nitrogen largely depends on the proper selection and combination of cryoprotectants. Often before freezing using DMSO aminobutyric acid, glycerol and various sugars, amino acids that perform cryoprotective function.

3. **Cooling rate.** In the classical method, chilled cryoprotectants added slowly to pre-chilled respective cultures. With experience, the most successful cryopreservation occurs when the sample is slowly and gradually cooled at a rate of one to several degrees Celsius for 1 min.

4. **Maximum cooling of examples to transfer into liquid nitrogen.** Positive results were obtained for samples cooled in the range of 30-40 °C for 1 h.

5. **Temperature storage.** All temperatures. With the exception of the temperature of liquid nitrogen (-196 °C) and vapor of liquid nitrogen (-160 °C), is unsuitable for long-term storage of plant tissues.

6. **Temperature and speed thawing.** Most of the plant tissue retains most viable if rapid thawing in a water bath at 40 °C, the speed of which can be equal to 10 to several hundred degrees Celsius for 1 min.

**Recovery of cells after freezing**

This period is particularly important for cell cultures and protoplasts. Immediately after thawing remove the dill plant protectors and carry objects in standard conditions of cultivation. Apply gradual addition of standard culture medium after dilution and enrichment of its mannitol and sorbitol, cultivation in the form of droplets, the semi-nutrient medium.

3. According to some studies, up to 2040 per year will disappear from 20 to 75
species. So important is the adoption of international conventions on the protection and conservation of biodiversity as a whole, including plants.

The fundamental basis for the conservation of plant genetic diversity and its collection is because in many countries actively forming banks and databases of plants that combine into a single global information network.

Domestic and international experience has shown that to preserve the gene pool is to create the most reliable bank of plant genetic resources (genetic banks). Today around the world operate more than 1,300 genetic banks of plants. In Ukraine, the formation of the National Plant Genetic Bank started in 1992. Today worked out conditions cryopreservation, storage and restoration of viability of vegetative parts meristems of fruit, berries, potatoes, garlic, seeds with reduced capacity for long-term storage (corn, soy, hemp, flax, onions, peppers, eggplant, pollens (rye, triticale, maize, sunflower, fruit crops). developed equipment for freezing and thawing for Cryo-depositories, the optimum cryopreservation types of containers for different types and amounts of plant material.

Vegetable banks not only retain numerous species, but information about them. Because directories are created with electronic versions which can be found on the Internet. Genebanks also serve to improve the taxonomy of flora and testing new approaches to identification. For this purpose, genomic methods, namely the study of the nucleotide composition of the DNA.

Besides studying the envisaged:
- Morphology and ultrastructure;
- The need for growth factors and supply;
- Physiological and biochemical features of metabolic processes
- Protein polymorphism;
- Amino acid composition.

However, the most important function of collections and banks - a source of starting material for biotechnology.

**THEME 8. RECEPTION BIOLOGICALLY ACTIVE SUBSTANCES**

Pharmaceutical products, flavorings, spices, dyes, stimulators derived from higher plants are complex chemical structure and are known as natural compounds. They either can not or very difficult to synthesize chemically.

In the 50's of last century found that during culturing plant cells can accumulate various substances, the synthesis of which is typical for this type of plant. Cell cultures of some plant species are able to synthesize a variety of secondary metabolites at concentrations close to or even higher than intact plants. At present these high-performance, and transformed culture, which transferred genes provide a synthesis of the target product is widely used in the industrial production of bioproducts.

**Classification of metabolic products**

Metabolism of cells characterized by considerable intensity, rapid transport of nutrients into the cell.

Substances in plants are conventionally divided into primary and secondary
metabolites.

The primary metabolites are:
- low "building" blocks for biopolymers - amino acids, sugars, organic acids, vitamins, cofactors;
- intermediates constructive metabolism - organic acids threecarboxylic acid cycle, pentose phosphate path products.

Primary metabolites are synthesized in the cell in amounts sufficient for the synthesis of all cellular structures. It is desirable for industrial biotechnology formation of excess product associated with defects of metabolism or regulation.

Secondary metabolites are antibiotics, mycotoxins, pigments, phenolic compounds, glycosides, alkaloids, rubber and rubber, essential oils, hydroaromatic compounds.

Many compounds of secondary metabolism - a major physiologically active substances such as terpenoids, which play a crucial role in the processes of respiration and photosynthesis. Therefore, the term "secondary metabolism substances" is gradually replaced with the term "specialized substance exchange." Some of these substances largely determine the nutritional and taste a variety of plant products, many of which are widely used in medicine, food and cosmetics industry, technology.

**Cell culture as a producer of secondary compounds**

Growing cell cultures in large-scale fermenters to produce biologically active substances similar to the cultivation of microorganisms. Plant cells can be cultivated under controlled conditions on a nutrient medium composition, as opposed to growing plants in the ground, where they often experience uncontrolled biotic and abiotic environmental factors. Cultivation of plant cells in continuous fermenters receiving fresh material throughout the year regardless of the climatic and seasonal changes. For example, in standard growth conditions with 1 g of cells cultured in 6-month term can get more than 100 g cell biomass.

During the culturing of cells and tissues is possible to produce new substances in the process of adding to the cultured cells natural intermediates required for biosynthetic processes.

**Cellular Biotechnology for obtaining medicinal plants**

First tissue culture of medicinal plants received F. White in 1945 of pink periwinkle *Vinca rosea*, and in 1947. R. Gotre received tissue culture henbane black *Hyosciamus niger* and showed its ability to synthesize relevant alkaloids.

Further introduction to the culture *in vitro* of organs, tissues and cells of medicinal plants and their study developed increasing rate. At the end of 50-ies were installed capacity of cultured tissues and cells synthesize important compounds characteristic of intact plants. In particular, we show that tissue culture guayule *Parthenium argentatum* able to synthesize rubber insulated roots and callus culture of belladonna *Atropa belladonna* - an alkaloid atropine, which accumulates in the roots of plants in the wild conditions.

In 1967 was created by another group to study cultured tissues of medicinal plants in the Leningrad Chemical and Pharmaceutical Institute, where he continued to work on tissue culture of ginseng and some other members of the family *Araliaceae,*
various species of *Rauwolfia*, first - *Rauwolfia serpentina*.

Since 1961 intensive study of tissue cultures as producers of drugs began in the United States / University of Nebraska and Iowa /.

Due to the high value pharmaceutical steroid hormones established perspectives of callus and suspension cultures for their production.

Revisions to the culture medium of arginine and ornithine enhances the growth of cultural roots of belladonna *Atropa belladonna* and increases it alkaloid content by 60%.

For example tobacco for the first time in 1942 was found the culture of isolated roots by nicotine biosynthesis. However, in callus cultures of different species and varieties of tobacco or this or other alkaloids hardly synthesized in the research of many authors. It was only in the 70s Japanese scientists Tabata, Furuya it was found that the biosynthesis of nicotine and other alkaloids can be regulated in callus tissues of any, including corn, origin exogenous growth promoters.

On the biosynthesis of secondary metabolites affect tissue of origin, duration of cultivation *in vitro*, mineral composition of the medium, the level, type and value of exogenous growth regulators and physical factors.

One of the first studies was the ability of cultured carrot cells accumulate carotenoids isolated mutant cell clones, characterized by inhibition of the synthesis of chlorophyll and a significant increase in the synthesis of carotenoids.

In the early 70-ies of XX century it was obvious that *in vitro* cultured cells of higher plants can be used in production as a source of alkaloids, enzymes, glycosides, essential oils, which led to the first patents in the design of bioreactors (fermenters) to obtaining large quantities of cell biomass. Among the first such work to be call a method of growing cells in liquid medium in culture vessels with a capacity of 20, 50 and 135 liters (dm³), mixing and aeration in which the environment is blowing sterile air. In 1976, Japanese researchers as a result of large-scale cultivation of biomass were tobacco cell volume of 20 m³.

First production cell biotechnology medicinal plants was receiving biomass callus tissue culture of ginseng, which was begun in the factories of the USSR in 1972. Industrial strain of ginseng callus culture BIO-2 was created based on callus derived by Butenko in 1960.

Cellular Biotechnology of medicinal plants thriving in different countries on an industrial scale is obtained ubiquinone, anthocyanins, alkaloids, glycosides.

In Japan, in 1990, received the following substances in excess of 90 million dollars of USA, in 1995 - more than $ 250 million USA.

**Features of accumulation of biologically active substances in vitro culture**

Despite several advantages, there are many reasons that hinder the production of biologically active substances in bioreactors. Most of organs or tissues that produce, live plants related substances as a result of their introduction to the culture did not synthesize these substances and synthesize them in small quantities. Need lasted selection work to culture was able to achieve an optimal level of biologically active substances.

Suspension cultures of plants are made up of aggregates of cells of different size. This means that the cells on the surface of the unit and in its center are not
identical, which makes the optimization of the production process are recycled - bioproducts. Some cell cultures convert sucrose to extracellular polysaccharides that enhance the aggregation of cells.

Found that production of the second product in the right quantity is often associated with the induction of organogenesis cell culture.

Secondary products in most crops do not stand out in the environment and remain inside the cells. It is characteristic of cultures that synthesize alkaloids and difficult extraction. Often, extraction and purification methods differ from the methods used for natural herbal products. Due to the large size of cultured plant cells sensitive to mixing and oxygen supply.

This requires special design fermenters (bioreactors).

Preparation of cell cultures for the production of biologically active substances is possible from any tissue or organ of the whole plants grown in the open or closed ground (Pic.18). Often this is used sterile seedlings obtained from seeds in vitro.

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Pic. 18. Scheme of receiving suspensial culture for production biological substances in fermenters
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To improve performance, widely cultured cells and in many cases successfully apply:
- cell breeding that is based on both spontaneous and induced by various mutagens in the variability of cultured cells;
- optimize growing conditions and storage growth and productive culture media;
- culturing differentiated cultures, cells or induction of differentiation, the use of elicitors.

Recently, in order to apply this method of genetic engineering. Highest scoring is the transformation of cells by bacteria Agrobacterium rhizogenes and a so-called bearded roots, whose performance is significantly higher than normal undifferentiated cultures. Increase synthesis of secondary metabolites by enhancing...
the relevant enzyme. This is possible:
- by introducing a heterologous gene with the same function of microbial or other species;
- substitution of its own gene promoter is stronger;
- introduction of the gene encoding enzymes insensitive to inhibition or gene encoding an antibody against the enzyme, which is a competitor for the same substrate and the desired gene;
- reduce catabolism secondary target compounds.

The task of future research that could transform biotechnology medicinal plants and herbal medicines for routine industrial technology, there is an in-depth study of the genetics of secondary metabolism.

**Cultivating of productive cell strains**

Plants and callus culture cells cultured under sterile conditions on special agar or liquid nutrient media in strictly controlled conditions. The fundamental approach is the possibility of formation of primary callus that initiates cell-suspension culture. At this level usually perform the assessment and selection of highly productive cell lines that continue to go deep cultivation in fermenters. Analysis of crops should be conducted to 3-4 th passage. Depending on the duration of one culture passage can vary from 15 to 45 days.

Differentiated tissue culture divided into cultural roots, buds, leaves and somatic embryos.

In determining the productivity of cell cultures, optimizing their growth and genetic stability leading place belongs to the culture medium. Their major components are plant hormones. From the balance of auxin and cytokinin, which are major components phytohormonal largely depends on the ability of cultures to synthesize this metabolite.

**Regulation of synthesis of secondary compounds**

By studying the synthesis of secondary metabolites in plant cell culture has accumulated a wealth of information, which indicates the existence of such laws:
- cultured cells are able to synthesize almost all classes of compounds of secondary metabolism (alkaloids, steroids, terpenoids, etc.);
- primary culture cells often contain a small number of specialized exchange or compounds do not contain them at all, but the contents of these compounds can significantly improve the optimization of culture medium, cloning, mutagenesis, etc.;
- synthesis of secondary compounds improves when slowing or suspension culture growth;
- in many cases, the synthesis of secondary compounds starts only in the case of differentiated in cell culture (morphogenic) structures;
- stability of the synthesis of secondary compounds varies for different classes of substances and for different cell cultures: synthesis of steroid glycosides are stable, whereas the synthesis of many types of alkaloids unstable;
- for cultured plant cells are characterized by an increase in the spectrum of the synthesized compounds specialized metabolism compared to intact plants, in some cases, while there is a synthesis of substances that are not typical of intact plants;
secondary metabolic compounds in plant cell culture is often characterized as the retrogressive changes in ontogenetic and phylogenetic in direction.

Given these patterns are established cell strains by obtaining adequate genotype (gene pool) cell populations capable of highly efficient synthesis of the desired compounds and full implementation of this resolution.

The technology involves the following steps:
• commute donor plant species, different species have varying capacity for synthesis in cell culture of the target substances, such as different types of poppy IP vitro with varying potential ability to synthesize alkaloids;
• commute highly specific plant-donor;
• genetic manipulation of tissue culture, including obtaining mutants co-makloniv and other cell selection approaches aimed at obtaining high-genetically modified strains;
• development of culture medium, conditions and methods of cultivation of genetically optimal for capacity due to the synthesis of the target substances;
• impact on growth (proliferation) of cells in culture to suspension or slowdown, which changes the cell metabolism towards the synthesis of compounds of specialized metabolism, for example, successfully used for this purpose, inhibitors of transcription and translation;
• search for signals by which plants occurs in intact control synthesis of secondary metabolites in cells (elicitors, nonspecific stressors, etc.);
• obtaining transformed by agrobakterii crops, such as "bearded roots» (hairy roots), which in many cases facilitates cultivation conditions and increases the content of secondary metabolites;
• obtaining transgenic crops to the synthesis of the desired product.

Selection of high cell strains

In order to increase productivity of cell cultures using different methods of selection.

Step selection. Stable clones were obtained by high-performance multiple selection callus or suspension cultures on special media with gradually increasing concentrations of the selective factor. So could increase, for example, the number of cells aymalinu Rauwolfia at 3-4.

Single-celled clones. Applied microbiological cloning techniques using single-cell suspension cultures of origin or culture protoplasts. This method is based on polymorphism for power output cells synthesize secondary products. Mini Colony, different higher accumulation of relevant secondary product selected visually, if the product is colored or by ELISA if the product unpainted. Thus, the selection of pigmented colonies of Fieldfare red nokorenevoho received line containing shikonin 25 times larger than the original strain.

Cell mutagenesis. The degree of variability of initial cell clones in their ability to synthesize secondary organic products increases with chemical or physical mutagens. In this way, high-performance strains obtained Rauwolfia serpentina dioskoreyi, Stephanie and other crops.
**Somatic hybridization.** Protoplast fusion provides new opportunities to improve and expand the range of cell lines capable of accumulating several secondary products or products previously unknown.

**Genetic Engineering.** This is one of the most effective methods for highly clones by introducing into the cell an alien information.

**Induction of secondary metabolism in plant cell culture.** Some microorganisms or their metabolic products during the joint cultivation of plant cells stimulate the synthesis of secondary metabolites. This process is known as elicitation is the response of plant cells to the joint cultivation as damage to plants pathogens leads to the synthesis of secondary products with antimicrobial properties.

**Cryopreservation of luxury cell lines.** From a practical point of view it is important to high-performance cell lines were maintained for a long time without changing their ability to synthesize secondary products.

**THEME 9. PROBLEMS OF ECOLOGICAL SAFETY**

One of the results of modern biotechnology, genetically modified organisms (GMO), particularly genetically modified plants (GMP). Release into the environment of genetically modified living organisms began in 1996 in the U.S.A. Acreage under these plants in the world are increasing: in 1996 GMO grown on an area of 1.7 million hectares in 1999 - 39.9, in 2002 - already at 58.7 million hectares. In 2002, crops were concentrated in the United States of America (39.3 million hectares), Argentina (13.5 million ha), Canada (3.5 million hectares). In these countries, genetically modified plants are grown in Australia, Bulgaria, China, Mexico, Spain, France, Germany, South Africa, Portugal, Romania and other countries. Most plants contain in their genome genes of microorganisms that ensures tolerance to a wide range of herbicides and resistance to pests. In 2003 created GMO more than 80 species, such as corn, oats, sorghum, rice, potatoes, cotton, soybeans, fruit. Among genetically modified crops largest areas occupied in 2002 soybean crop (36.5 million hectares), maize (12.4 million hectares), cotton (6.8 million hectares), canola (3.0 million hectares).

The main goal of the research is using of agricultural crops and to establish the possibility of transferring genes embedded in other organization body naturally environment, impact GMO resistant to pests.

The feasibility of the use of genetically modified organisms in the practice of rural economy-one, each country decides on the basis of its own legislation and analysis of the security environment.

Was developed and agreed at an international meeting in Montreal in January 2000 "The Cartagena Protocol on Biosafety". It was signed by 68 members of the European Union, Bulgaria, Belgium, Finland, France, Germany, Italy, Lithuania, the Slovak Republic, the Czech Republic and Hungary.

"The Cartagena Protocol on Biosafety" is aimed at ensuring proper discharge on the level of protection in the field of the safe transfer, processing and use of genetically modified organisms that may adversely affect the conservation and sustainable use of biological diversity, taking into account risks to human health. This
document obliges countries to ensure that genetically modified organisms, processing, transport, use, transfer and release of the environment in order to prevent or significantly reduce the potential risk of a breach of biodiversity and deterioration of health.

Transfer of genetic material into related organisms in vivo may occur during the propagation of plants. During the preliminary studies, usually genetically modified plants are grown in greenhouses, "in sterile conditions," it is difficult to predict the consequences of transgenic plants in the wild. Conducting biochemical analysis sometimes does not give a complete picture of the processes of introduction of gene material, because it always allows you to record the body built of gene expression products due to poor sensitivity of some methods. Detection method is a method of polymerase chain reaction (PCR), by means of which one can assess the presence or absence of a particular nucleotide sequence in the genome of genetically modified plants.

Status of environmental security report shows a group of leading scientists in the world under the leadership of Nobel laureate D. Kendella. The report "Bioengineering of crops: report of the World Bank transgenic crops" allegedly created bioengineered methods of varieties in principle no more dangerous to the environment than traditional.

In Ukraine testing genetically modified plant varieties held from 1997 to 2000 experienced a sugar beet resistant to Roundup herbicide, corn resistant to corn borer and Roundup herbicide, Basta. Due to unregulated by law for trial registration standards and safe use of transgenic plant varieties and hybrids conveyed lshe test all GMO suspended.

Risk assessment of transgenic plants

Often considered the impact of transgenic plants on the environment else that may occur is that:

• constructed genes are transferred from the pollen wild species and their hybrid offspring receive properties increased seed product arrangements or ability to compete with other plants;
• transgenic agricultural plants become weeds in agricultural and displace other plants that grow close;
• transgenic plants will be a direct threat to humans, domestic and wild animals.

Studies show that environmental risk in the event of cultivation of transgenic plants is comparable to the risk of testing new varieties breeding. All compounds detected in transgenic plants already exist in nature.

Phytoremediation

One of the new areas of transgenic plants is their use for phytoremediation - clean soil, groundwater against pollutants: heavy metals, radionuclides and other hazardous compounds.

Found that mercury resistant bacteria expressing the gene merA who co-blowing protein transport and detoxification of mercury. Modified design of gene merA used to transform tobacco, rape, poplar, arabidopsis.

Heavy metals are among the pollutants of agricultural land. It is known that
most plants accumulate cadmium in the roots, while some plants such as lettuce and tobacco, it mainly accumulate in the leaves. Cadmium enters the soil mainly from industrial emissions and phosphate fertilizers as an impurity.

One of the approaches to reduce the flow of cadmium in humans and animals is a transgenic plant leaves are used as food or animal feed rynam and which accumulate less of the metal in the leaves. One solution to this problem is to provide plants with increased synthesis in roots fitohelatyniv - natural cadmium-binding peptides found in plants.

**Environmental productions in biotechnology**

Increasing anthropogenic pollution of the biosphere leads to interference in the process of self-purification, as a result of environmental disasters torn natural connection between the various elements of ecosystems. This affects the composition of microbial communities as a component of the process of circulation of elements in nature. To restore the integrity of biological systems to recover completely or partially lost its components.

World experience shows that appropriate and environmentally sound method of protecting the environment is biotechnology. It involves use of a process based on the use of microorganisms for the disposal of various waste industry and agriculture recultivation of land contaminated by various toxic compounds, wastewater treatment. Biotechnology is an effective natural way to combat pollutants of soil, water and air, because it is based on the methods of wildlife that use of specific mechanisms of degradation and recycling of toxicants.

To solve environmental problems often resort to industrial microbiology as:

- bacteria synthesize useful substances to man and do it hundreds of times faster than a plant or animal, such substances include proteins, amino acids, vitamins, enzymes and other organic compounds;
- biotechnological processes are carried out in special plants for use of environmentally sound technologies;
- companies can operate in any geographical area, including the abandoned lands;
- a significant number of agricultural waste and other economy is the raw material for microbiological production.

**Biological plant protection**

Microorganisms natural ecosystems, according to biological laws, normally can not unbalance the past, as are their constituents, or product. Therefore, biological plant protection products based on microorganisms have advantages over chemical due to environmental safety.