Bacteria – Plant Interaction:

Transgenic Roots for Human Health

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ABBREVIATIONS

SANBI – South African National Biodiversity Institute
Ti-plasmid – Tumor induced plasmid
vir genes – virulence genes
rol genes – *root loci* or *root locus* genes
T-DNA – region (area) of transport DNA (T-DNA), which is transferred to plant cells by the action of virulence genes (*vir*) from the same plasmid
IAA – indoleacetic acid
orf – open reading frame
mis – mikimopine synthase sequences
iaaH – indole-3-acetamide hydrolase
Acs – agrocinopine synthase
CFU – coliny forming unit
DMBQ – 2,6-dimethoxy-1,4-benzoquinone
UV light – Ultraviolet light
LB – left border
RB – right border
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
Cas9 – CRISPR-associated nuclease
MSC – multiple cloning sites
Tra/trb – transcriptional activators
B marker – bacterial selectable marker
Pro – promoter
Rep – reporter gene
FDS – farnesyl diphosphate synthase gene
GFP – green fluorescent protein
ifn-α2b – human interferon-α2b
SMs – secondary metabolites
SH – Schenk & Hildebrandt medium
MS – Murashige & Skoog medium
B5 – Gamborg medium
SOD – superoxide dismutase
GPX – glutathione peroxidase
PAL – phenylalanine ammonia-lyase gene
CHI – chalcone isomerase gene
FLS – flavonol synthase gene
DPPH – 2,2-diphenyl-1-picrylhydrazyl
ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ZFNs – zinc finger nucleases
NLS – nuclear localization signal
p35S – Cauliflower Mosaic Virus promoter
GUS FW *β*-glucuronidase, a product of the *uid*A gene, which converts a soluble colorless substrate 5-bromo-4-chloro-3-indolyl-*β*-*D*-glucuronic acid into an insoluble colored product chloro-bromoindigo
FW – fresh weight
RSA – radical scavenging activity
CTAB – cetyltrimethylammonium bromide
AgNPs – silver nanoparticles

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ABSTRACT

Plant genetic engineering as a group of technologies and methods that lets scientists change the DNA of many organisms, including plants, bacteria, and animals, covers such methods as biolistic transformation, microinjection, *Agrobacterium*-mediated transformation. The monograph includes the information about some aspects of the genetic transformation of plants using *Agrobacterium rhizogenes* soil bacteria: characteristics of the bacteria, role of Ri-plasmids in horizontal gene transfer in nature, plant – *Agrobacterium* interaction, effect of *rol* genes on plant secondary metabolism etc. There is also the data about hairy roots induction, their characteristics, cultivation and using as producers of valuable bioactive compounds.

The book is designed for a wide range of specialists in the field of plant biotechnology, genetic engineering, biochemistry, and plant physiology. The book can be used by students of biological and agricultural faculties as a study guide and a source of information for the preparation of essays and dissertations. It can also be of interest to all readers who want to have knowledge of new achievements in biological science and their practical application.

KEYWORDS

Bacteria-plant interaction, genetic engineering, plant biotechnology, hairy roots, human health, bioactive compounds.

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PREFACE

In this book, you can read about some aspects of the interaction between plants and microorganisms and the possibility of using such interaction. The plants surround us everywhere. They are near your house. They greet you every morning when you look out the window. They accompany you on your way to the university or office. They welcome you on your day off when you are relaxing. Plants give people oxygen and food, clothes, feed, and energy.

You know that microorganisms are all around us. These are small inhabitants of our planet. There are many of them around, but we often do not even guess they are our neighbors. They are very different. Some microorganisms are friendly to humans. They can also be friends with plants. These miniature creatures help plants grow, survive natural disasters, and increase productivity. People have learned to use "friendly" microorganisms for themselves and plants.

You will learn what hairy roots are, why they are hairy, how they are formed, and how they can be used for human benefit. You can see that they are very different, but all of them are a source of valuable biologically active compounds and can be used in treating of people.

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CONTENT

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INTRODUCTION

Plant genetic engineering is one of the modern directions in science today. It involves the possibility of using various tools of plant genome changing. For example, such methods include biolistic transformation, the microinjection method, as well as the use of bacteria of the *Agrobacterium* genus.

The last method includes the cocultivation of plant parts (leaves, stems, roots, internodes, petioles, and even flowers) with a suspension of *Agrobacterium tumefaciens* and *A. rhizogenes*. These bacteria are soil microorganisms that have a natural ability to infect plants and transfer part of their genome (specific genes) to the plants. Thanks to such features of these microorganisms, it was possible to develop a method of plant genetic transformation.

A. tumefaciens are used to obtain transgenic plants, and *A. rhizogenes* are used to obtain transgenic roots (so-called hairy roots). Hairy roots can be cultivated for an unlimited long period, synthesize plant-specific compounds in an amount that significantly exceeds the amount in the mother plants, and synthesize compounds of mammalian or bacterial origin by transferred genes.

Works using genetic engineering are aimed to change plants to increase their resistance to the effects of negative factors of the environment. In addition, this method allows to change the metabolism of cells, stimulates the synthesis of specific plant components or compounds that are not typical for plants. Thus, transformed plants or hairy roots can be a source of compounds with medicinal properties and used as pharmaceutical biofactories. That is why research on the genetic transformation of medicinal plants that synthesize biologically active compounds can attract special attention.

The work of the authors of this book for many years was aimed at developing methods of genetic transformation of medicinal plants to obtain hairy roots, make a collection of transformed plant roots of various species, conduct a comprehensive fundamental study of the collection samples to find out the specifics of the effect of transformation and gene transfer on the functioning of cells plants, as well as on the development of methods for obtaining hairy roots with a high content of valuable compounds for their possible use as bioproducers of medical compounds.

The collection of hairy roots includes such species *as Artemisia annua, A. vulgaris, A, dracunculus, A. tilesii, Althaea officinalis, Cichorium intybus, Bidens pilosa*, and others (https://plants.usda.gov/). The conducted studies were related to the determination of the features of the functioning of the plant antioxidant protection system (for example, the activity of the superoxide dismutase enzyme), the level of antioxidant and reducing activity, the synthesis of such compounds as flavonoids, sugars, artemisinin. The research was also focused on the possibility of using extracts from hairy roots (for metal nanoparticle synthesis, in particular).

Such studies using a unique collection of hairy roots make it possible to comprehensively assess the influence of genes transferred during transformation on the functioning of plant cells. In addition, such studies make it possible to obtain samples of hairy roots as a result of genetic transformation, which can be used as a source of a complex of biologically active compounds with medicinal properties for their use in the pharmaceutical industry.

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1. THEORETICAL BACKGROUND AND CURRENT STATE OF PLANT GENETIC TRANSFORMATION RESEARCH AND SELECTED TRENDS OF PRACTICAL APPLICATION

1.1. *Agrobacterium rhizogenes*: role in nature and use in biotechnology

Attempts to understand the possibility of plant transformation date back almost a century. This became possible thanks to agrobacteria capable of transferring their T-DNA into the plant genome. Thus, agrobacterial transformation initiated the genetic engineering of plants as such. It is the oldest and the most common method of plant transformation, which remained almost unchanged since the 1970s, because the technique is very simple and easy to reproduce.

The history of agrobacterial transformation can be presented in the following stages (Somssich, 2019):

a) research on crown gall disease (1892 – 1966);
b) determination of the meaning of agrobacterial transformation (1967 – 1976);
c) attempts to create the first transgenic plants (1977 – 1986);
d) the beginning of biotechnology as an industry (1980 – 2005);
e) development and simplification of biotechnological methods (1987 – now).

At first, scientists noticed the fleshy outgrowths on the crown roots of different fruit trees in the late 19th century. The name "crown gall" was chosen in 1892 to describe these tumor-like outgrowths, and Erwin Smith was the first scientist to speculate that bacteria could be the cause of these tumor formations. He published his review on the current state of knowledge in the field in 1896. However, the idea that bacteria could infect plants was seen as outrageous and non-scientific by many reputable experts in microbiology at the time, such as Alfred Fischer. Despite that, Smith continued his research and published new data to support his idea in 1907 and 1912. Again, he was the first scientist to describe the causative agent of what he called "plant cancer" – *Bacterium tumefaciens*. Alas, this idea was not readily accepted within the field as well. At the same time, another plant disease was studied – a hairy root disease (Srivastava & Srivastava, 2007). Its name was first introduced in the literature by Steward *et al.* in 1900. Later, Riker *et al.* (1930) described and named its causative microorganism *Phytomonas rhizogenes*, which was later renamed *Agrobacterium rhizogenes*.

The next breakthrough came in 1941 when Philip White and Armin Braun demonstrated the possibility to culture explants from crown gall tumors. While these explants retained a tumor-like growth, scientists were unable to isolate the causative bacteria, *Phytomonas tumefaciens*, from the tissue (final re-classification as *Agrobacterium tumefaciens* was in 1942). Armin Braun speculated that DNA might be involved, and continued his research over the next 30 years. He established tumor lines growing on hormone-free medium for decades. His pioneering work earned him the title "Godfather of Crown Gall Research". However, the nature of plant transformation in the 1940 – 1960s was still unknown.

In 1967 Rob Schilperoort and colleagues synthesized a short RNA strand from a complementary agrobacterial DNA sequence they had isolated from a cultured *Nicotiana tumor*. This DNA sequence was found only in *Agrobacterium tumefaciens*, but not in healthy *Nicotiana* plants that indicated that bacterial DNA had indeed been transferred into the plant cell. Other scientists, such as Jeff Schell Marc van Montagu, Mary-Dell Chilton, and Allen Kerr continued the research. The Schell/van Montagu lab published an article in 1974 that was the first major contribution on the way toward identifying the "tumor-inducing principle". They identified the large plasmid that could only be found in oncogenic tumor-inducing *A. tumefaciens* strains. Next year, they transferred the plasmid to a non-oncogenic *Agrobacterium* strain and demonstrated that this strain gained the ability to induce tumors. They called this plasmid Tumor-inducing (Ti)-plasmid.

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Simultaneously, *A. rhizogenes* was studied. Its ability to induct the hairy roots was accepted and supported by many researchers. The first directed transformation of higher plants (*Nicotiana tabacum*) using *A. rhizogenes* was made by Ackermann in 1973 and published in 1977 (Ackermann, 1977).

In 1980 Schell and van Montagu concluded that only a region of Ti-plasmid DNA is incorporated into the plant genome. This region was called T-DNA (Transport-DNA). In 1981, the Schilperoort, Schell/van Montagu, and Nester labs all published on Ti-plasmid mutants carrying insertions in different regions of the T-DNA. In 1983, Chilton lab published the successful regeneration of healthy *Nicotiana tabacum* plants carrying a full-length engineered agrobacterial T-DNA. Thus, the race ended and genetic engineering of plants via *Agrobacterium*-mediated transformation started all over the world.

Since then, both species of agrobacteria are used for the genetic transformation of plants and are called "natural genetic engineers" due to their ability to incorporate T-DNA of Ti- (tumor-inducing) or Ri- (root-inducing) plasmid into the plant genome (Fig. 1). Obtained crown galls and hairy roots are the formations with characteristic appearance (see PlantDiseases.org. Hairy root and crown gall diseases) and ability to produce opines (their synthesis is coded by genes included in T-DNA) – amino acid derivatives that agrobacteria "feed on". That is why these bacteria are phytopathogens in nature.

In *Agrobacterium*-mediated transformation, each incorporations of T-DNA into a plant cell is an independent transformational event. Thus, using this method of genetic engineering, it is possible to quickly obtain a broad spectrum of new transformant lines with enhanced or completely new characteristics, different from control plants. Moreover, hairyroot cultures show rapid biomass increase, growth on hormone-free media, and enhanced secondary metabolism due to transferred *rol* genes. For an even greater yield of the metabolites of interest, it is possible to change the metabolic pathways of the biosynthesis of compounds by transferring additional genes and CRISPR/Cas methods. In addition to secondary metabolites, recombinant proteins are also obtained in this way today. All this information will be discussed in this chapter of the book.

1.2. *Agrobacterium rhizogenes* in the nature

Agrobacterium rhizogenes (Riker et al. 1930) Conn 1942 is a member of the Alphaproteobacteria class, Rhizobiaceae family. Its homotypic synonyms (NCBI database, 2023) are *Rhizobium rhizogenes* (Riker et al. 1930, Young et al. 2001), *Agrobacterium* genomic group 10, *Agrobacterium* genomic species 10, *Agrobacterium* genomosp. 10, *Agrobacterium* biovar 2. The latter is related to the alternative special purpose nomenclature (Garrity, 2005). It involves naming strains according to their pathogenic character, such as *A. tumefaciens*, *A. rhizogenes*, or *A. radiobacter* for tumorigenic (carrying Ti plasmids), rhizogenic (carrying Ri plasmids), and nonpathogenic strains, respectively, and therefore classifying them as *Agrobacterium* biovar 2.

The genus name "*Agrobacterium*" is comprised of two parts: "*agros*" (Greek noun "a field") and "*bakterion*" (Greek noun "a small rod"), i.e. "a small field rod". Another genus name "*Rhizobium*" is comprised of two parts as well: "*rhiza*" (Greek noun "a root") and "*bios*" (Greek noun "life"), i.e. "living in a root". The species name "*rhizogenes*" is comprised of two words: "*rhiza*" (Greek noun "a root") and "*gennao*" (Greek verb "to make, to produce"), i.e. "root-producing". It is an intrinsic feature of these bacteria as carriers of Ri-plasmids, which, in turn, cause hairy root disease in wounded plants. Pathogenic *A. rhizogenes* strains have a wide, and perhaps complex, host range that will be discussed further.

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The status of *A. rhizogenes* as an authentic species in the genus *Rhizobium* is supported by numerical analysis of nutritional and biochemical data and comparative 16S rDNA sequence analysis (Garrity, 2005).

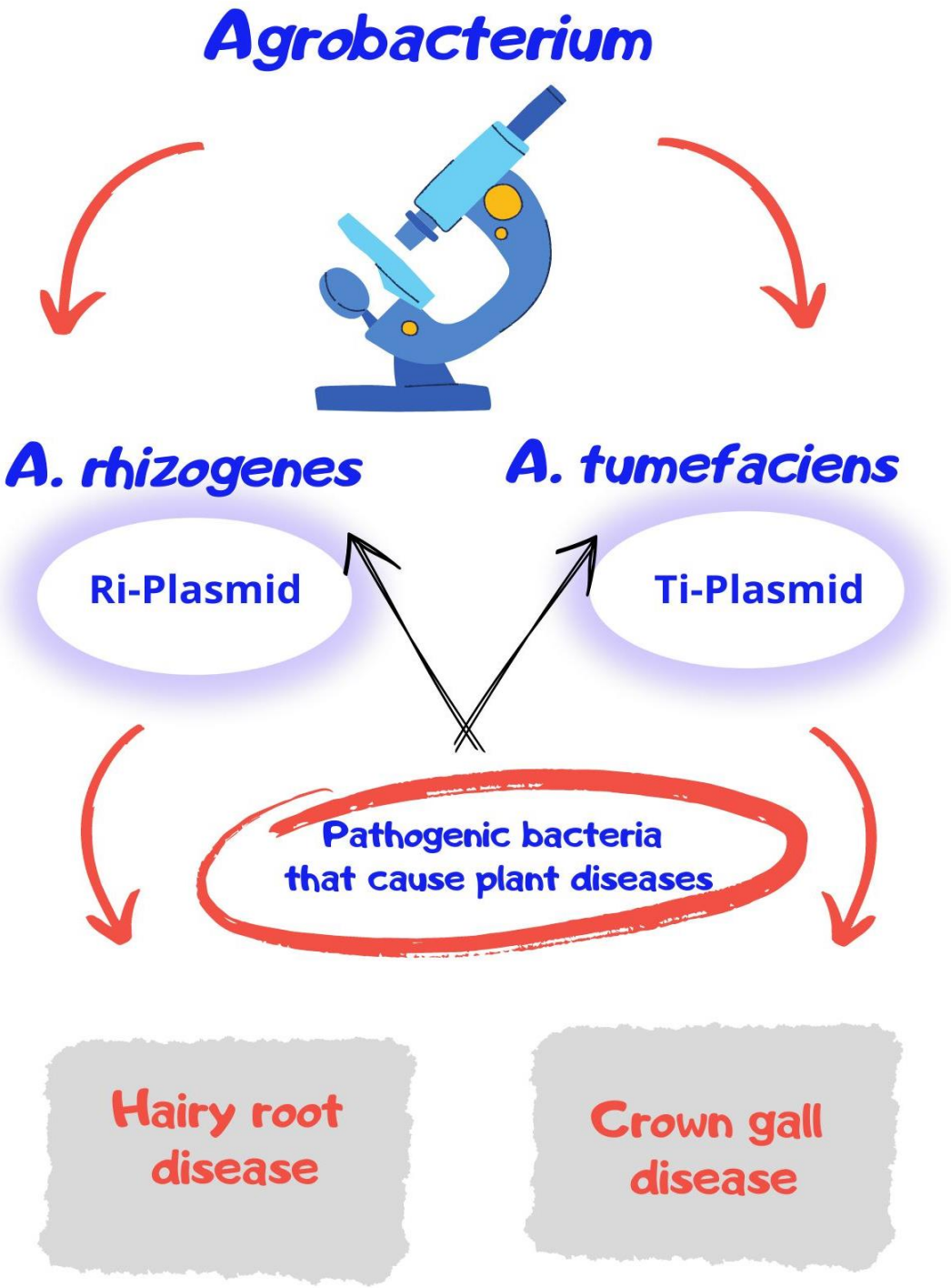

Figure 1 – *Agrobacterium rhizogenes* and *A. tumefaciens* can cause plant diseases

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1.3. Characteristics of *Agrobacterium rhizogenes* from Bergey Manual of Systematic Bacteriology (Garrity, 2005, Volume 2, part C)

General characteristics. *A. rhizogenes* are Gram-negative aerobic nonsporeforming bacteria in a rod shape, 0.5–1.0 x 1.2–3.0 μm in size (see SANBI site – South African National Biodiversity Institute. *Rhizobium rhizogenes*). They are motile and have 1–4 peritrichous flagella. The optimal temperature for growth is 25–28°C; they do not grow at a temperature above 30°C. The optimal pH for growth is 5–9; possible range is pH 4–10. Generation times of *Rhizobium* strains are 1.5–5.0 h. Colonies are usually white, light beige or non-pigmented, circular, convex, semi-translucent or opaque, raised and mucilaginous, usually 2–4 mm in diameter within 3–5 days on yeast-mannitol-mineral salts agar. Growth on carbohydrate media usually is accompanied by copious amounts of extracellular polysaccharides.

It is a chemoorganotrophic bacterium, utilizing a wide range of carbohydrates, salts of organic acids, and amino acids as sole carbon sources, without gas formation. The bacterium does not produce 3-ketolactose (this feature differentiates it from *A. tumefaciens*). It utilizes a relatively wide range of organic substrates as sole sources of carbon: l-arabinose, d-cellobiose, d-fructose, d-galactose, d-glucose, l-histidine, inositol, d-mannose, rhamnose, ribose, sorbitol, d-turanose, and d-xylose. It also can utilize l-arginine, amygdalin, l-citrulline, erythritol, ethanolamine, l-lysine, methyl-d-glycoside, l-ornithine, propionate, and sarcosine, which differentiates it from other agrobacteria. Cellulose, glycine, glycogen, inulin, oxalate, urea, and starch are not utilized. The bacterium produces an acidic reaction in mineral-salts medium containing mannitol or other carbohydrates; requires biotin as a growth factor (*A. rhizogenes* strains do not utilize nitrate unless biotin is supplied; some strains require both l-glutamic acid and biotin); does not grow in media containing 2% NaCl. Ammonium salts, nitrate, nitrite, and most amino acids can be nitrogen sources. Peptone is poorly utilized. Casein, starch, chitin, and agar are not hydrolyzed by this bacterium.

The mol% G–C of the DNA is: 59–63 (*Tm*).

Type strains: ATCC 11325, DSM 30148, ICMP 5794, IFO 13257, LMG 150, NCPPB 2991.

GenBank accession number (16S rRNA): D01257, D14501.

Cell wall composition. The cell wall structure of *Rhizobium* is similar to that of other Gram-negative bacteria. The peptidoglycan consists of glutamic acid, alanine, diaminopimelic acid, and amino sugars. In addition, several pathogenic strains have leucine, phenylalanine, serine, and aspartic acid in the peptidoglycan layer as well. Lipopolysaccharide composition varies from strain to strain but usually contains 2-keto-3-deoxyoctanoic acid, uronic acids, glucosamine, glucose, mannose, rhamnose, fucose, and galactose. Rhizobia also has an unusually complex composition of membrane phospholipids, phosphatidylcholine is among them.

Fine structure. Cellulose-containing fibrils, which anchor the bacteria to the plant cell surface, are formed by pathogenic *Agrobacterium* strains during their attachment to plant cells at the wounded site.

Cultural characteristics. All bacteria produce abundant water-soluble extracellular heteropolysaccharides and glucans. Chromosomal genes termed *chv* are responsible for the production of cyclic glucans essential for virulence in all species of *Agrobacterium* (*Rhizobium*).

Plasmid-mediated plant-pathogenic (rhizogenic) activity in *A. rhizogenes*. Rhizogenic (hairy rot producing) activity of *A. rhizogenes*, as well as oncogenic (tumorigenic) activity of *A. tumefaciens*, are mediated by genes that are carried on one or more large (>150 kb) plasmids. Tumorigenic activity is conferred by Ti-plasmids and

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rhizogenic activity is conferred by Ri-plasmids. Rhizogenic genes on the Ri-plasmid comprise T-DNA genes and virulence (*vir*) genes. These last do not incorporate into the plant cell genome, although their presence is intrinsic to the successful transformation. *Vir* genes are activated by the phenolic compounds of wounded plant tissue, such as acetosyringone and lignin precursors. These genes facilitate the transfer of a T-DNA (8–22 kb) of Ri-plasmid that integrates into the plant nucleus genome in one or more copies. T-DNA carries *rol* ("root loci" or "rooting locus") genes, necessary for hairy root initiation and growth, as well as secondary metabolism activation and opine production. Opines are acid derivatives produced in hairyroots. These compounds are the nutrient source of carbon and nitrogen for agrobacteria and are not utilized by plants. That is why these bacteria are considered pathogenic. About 30 opines have been identified, the most common and well-known of which are octopine, nopaline, cucumopine, mannopine, and agropine (Zárate 2010; Trovato et al. 2018). The set of opines is unique for each strain and for each type of Ri-plasmid.

Pathogenic host range. The host range of rhizogenic *Agrobacterium* strains is very wide: De Cleene and De Ley (1981) reported 37 plant species belonging to 30 genera in 15 families of dicotyledonous plants as susceptible to transformation by *A. rhizogenes*. However, they investigated 250 monocotyledonous species, and none of them were susceptible to the disease, except some members of Liliales and Arales. Bradbury (1986) listed over 50 plant species affected by rhizogenic strains as well. Later, Porter (1991) reported that more than 450 species of many different genera and families are known to be susceptible to infection by *A. rhizogenes*. Since then many more additions have been made to the list, even among trees-gymnosperms, e.g. *Taxus* (Syklowska and Sygitowicz, 2019).

There have also been indications of narrow host specificity within some species (Anderson and Moore, 1979; Unger et al., 1985; Paulus et al., 1991) and within some populations of different countries. Such specificity is quite variable among *A. rhizogenes* strains (Paulus et al., 1991).

Indeed, monocotyledonous species are rarely transformed with *A. rhizogenes* due to several limitations (Hao et al., 2021): monocotyledons are not easy to produce phenols; their cells are prone to lignification, adverse differentiation, and selective response to *A. rhizogenes* strains. Nowadays, there are many more possible monocotyledonous hosts of *A. rhizogenes* due to the advances in biotechnology. In most cases, successful transformation and hairyroot initiation in monocotyledons and gymnosperms require the addition of acetosyringone or a usage feeder layer (Syklowska and Sygitowicz, 2019).

Agrocin activity. It was found that *A. rhizogenes* strain 84 (ICMP 3379; NCPPB 2407) can synthesize plasmid-encoded agrocin (New and Kerr, 1972). It is a toxic analog of an adenine nucleotide and selectively inhibits pathogenic *Agrobacterium* strains harboring a nopaline Ti-plasmid. This compound was studied as a tool for the biological control of crown gall disease and was found successful. Strain 84 is now available in commercial drugs as a biological control agent with wide application.

Ecology. *Rhizobium* occurs worldwide in soils and plant rhizosphere, although some unique species may be isolated from limited geographic regions due to the distribution of their hosts. *Agrobacterium* strains have also been reported in some human clinical specimens (CDC group Vd-3) (Rubin et al., 1985). They are usually nonpathogenic and occur either as incidental inhabitants in the patient or as contaminants.

Antibiotic sensitivity. In general, wild-type *Agrobacterium* species can be sensitive to chlortetracycline, gentamicin, neomycin, novobiocin, oxytetracycline, and tetracycline but are commonly resistant to nalidixic acid. Its growth is inhibited by low concentrations (3–780 μg/ml) of metacycline, doxycycline, sigmamycin, and triacetyloleandomycin.

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1.4. Ri-plasmids

A. rhizogenes plasmids are approximately 200 kbp (Kang et al., 2020). They have a region (area) of transport DNA (T-DNA), which is transferred to plant cells by the action of virulence genes (*vir*) from the same plasmid. The core *vir* genes and chromosomal virulence (*chv*) genes are essential for pathogenicity. *Vir* are located in four operons, in addition to *virA* and *virG* for a two-component regulatory system that receives signals from plants. The most significant *vir* genes are *virD1* and *virD2*, which portray proteins that attach to and cut DNA at 25-bp T-DNA border repeat sequences (Georgiev et al., 2012). Other *vir* genes, such as *virE1* and *virE2* are significant as well. Proteins translated from these genes shield T-strands from nuclease digestion and facilitate their integration into the plant chromosome. Interestingly enough, some *A. rhizogenes* strains do not possess these genes but still transfer T-strands effectively due to the pRi *GALLS* gene portraying a protein with a nuclear localization signal and helicase activity.

The T-DNA region(s) contain genes for the synthesis of phytohormones that induce excessive cell division in plants. T-DNA usually contains a set of *rol* genes (*rolA*, *rolB*, *rolC,* and *rolD*) that stimulate the formation of additional roots and enhance secondary metabolism. T-DNA of Ri-plasmids is randomly integrated into the plant genome and expressed as mRNA (Gutierrez-Valdes et al., 2020). TL-DNA and TR-DNA are usually independently transferred and stably integrated into the genome of the host plant. However, TL-DNA alone is vital and sufficient for the induction of hairy roots.

Strains can carry one, two, or three T-DNAs on their pTi/pRi plasmid (Chen & Otten, 2017). T-DNA is surrounded by direct repeats of approximately 25 nucleotides (so-called boundaries or borders). The transmission starts from the so-called right boundary and continues to the left boundary. Integrated T-DNAs are often incomplete and truncated in the left part. They can occur as single copies or as tandem or inverted repeats.

The complete genome sequences of various strains of *A. rhizogenes* are available in the literature. For example (Hooykaas & Hooykaas, 2021), the genome of strain LBA9402 (NCPPB1855rifR) consists of 3958212 bp chromosome, a 2005144 bp chromid (secondary chromosome), and 252168 bp Ri-plasmid (pRi1855). This agropine Ri-plasmid has about 4% lower GC content than the rest of the genome. It has two T-regions, one of which, the TL-region, contains *rol* genes. Ri plasmids of other strains may have only one T region with very similar genes (Otten, 2018). One (in cucumopin and mikimopin Ri-plasmids) or two (in mannopin Ri-plasmids) non-conservative genes are present at the very right end of the TL region. They encode cucumopine, mikimopine, and mannopin synthases, respectively.

This agropine Ri-plasmid, in addition to the conservative TL-site, has an additional TR-site containing *aux*-genes involved in the biosynthesis of auxin indoleacetic acid (IAA) (Offringa et al., 1986), and the genes *mas*1, *mas*2, *ags* for agropine biosynthesis (Bouchez & Tourneur, 1991). The virulence region of pRi1855 responsible for T-DNA transfer into plant cells contains the important virulence genes *virA*, *virB1-virB11*, *virG*, *virC1*, *virC2* and *virD1-virD5* in the same order as in other Ri and Ti plasmids, but the *virE1* and *virE2* genes are absent and replaced by a novel *orf* (Open Reading Frame) with some similarity to the nopaline pTi of *virF*.

Hairy roots formed via transformation with such agropine strains contain agropine, agropinic acid, mannopinic acid, and mannopine. Moreover, *A. rhizogenes* strains such as A4, but not NCPPB1855 contain a second, catabolic plasmid with genes for catabolism of the other three mannityl opines. Researchers (Hooykaas & Hooykaas, 2021) have identified the genes for agropine transport and catabolism in pRi1855, which are located in a segment of the plasmid adjacent to the TR region.

Until recently, agrobacteria were considered the only bacteria that can transfer part of their DNA (T-DNA) prom Ri-/Ti-plasmids to the plant genome. However, in 2022 Cho et

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al. published an article on a novel bacterium, *Ochrobactrum haywardense* H1 (Oh H1), which was discovered to be capable of efficient plant transformation. *Ochrobactrum* spp. are environmental organisms and are considered opportunistic pathogens of low virulence in humans (Wisplinghoff, 2017). *Oh* H1 is a unique, non-phytopathogenic species, which can host *Agrobacterium*-derived *vir* and T-DNA and helps to deliver transgenes in soybean. The researchers generated a cysteine auxotrophic *Oh* H1-8 strain containing a binary vector system. It generated high-quality transgenic events by single-copy, plasmid backbone-free insertion at frequencies higher than those of *Agrobacterium* strains (up to 35%). Scientists have demonstrated that *Oh* H1-8, combined with spectinomycin selection, is an efficient, rapid, marker-free, and yield-neutral transformation system for soybean, and hopefully, for other plants. Such novel findings are very promising and should be researched further.

1.5. Role of Ri-plasmids in horizontal gene transfer in nature

In all eukaryotes, including plants, the maintenance of the integrity of species is ensured by the vertical transmission of genetic information through reproduction. However, horizontal gene transfer (HGTs) exists as well. It is the interspecific transmission of genetic information across reproductive barriers that are very common in nature and proceeds through different agents: parasitic plants (*Orobanche*, *Cuscuta*, *Rafflesiae*, *Striga*), viruses (*Florendivirus*, *Totivirus*, *Pararetrovirus*, Cucumber necrosis virus), bacteria (*Agrobacterium*) and fungi (*Colletotrichum*) (Aubin et al., 2021). Nowadays, the advent of next-generation sequencing technologies has opened new perspectives and possibilities for the study of HGTs through comparative genomic approaches.

The agrobacterial T-DNA incorporation mechanism is HGT-dependent *sensus stricto*, as it is the transmission of genetic material from bacteria to plants. However, as it turned out, many plants in nature carry some regions of agrobacterial DNA (similar to the phenomenon of plants carrying some regions of viral genetic material) that are inherited transgenerationally without any visible change in plant morphology, i.e. acquisition of crown galls or hairy roots. This occurrence was initially reported in *Nicotiana glauca* (tree tobacco), which carries in its nuclear genome a region homologous to the part of the Ri-plasmid of *A. rhizogenes* (White et al., 1986). This region of plant DNA was called cellular T-DNA (cT-DNA) and was initially described as an imperfect inverted repeat that contained two homologs to *rol* genes, *NgrolB* and *NgrolC* (Ng, *N. glauca*). Later, the cT-DNA was found to contain two additional genes corresponding to open reading frames *ORF13* and *ORF14* and mikimopine synthase (*mis*) sequences (*NgmisL* and *NgmisR*) that indicated that its mikimopine-type Ri-plasmid origin (Quispe-Huamanquispe et al., 2017).

Intrieri and Buiatti (2001) screened 42 *Nicotiana* species and found that at least one of genes *rolB, rolC, ORF13,* and *ORF14* genes were detected in the genome of 15 species. Their phylogenetic analyses concluded that the *rol* genes seemed to follow the evolution of the *Nicotiana* genus via more than one independent infection by *A. rhizogenes* in ancient times. This hypothesis was supported through deep sequencing of the genome of the ancestral tobacco species *Nicotiana tomentosiformis* (Chen et al., 2014). The genome of *N. tomentosiformis* contains four cT-DNAs (TA, TB, TC, and TD) all of which are derived from different *Agrobacterium* strains. Each of these cT-DNAs contains an incomplete inverted-repeat structure. The TB region contains an intact mannopine synthase 2′ gene (TB-*mas2*′) that is highly expressed in the roots of some *N. tabacum* cultivars. These results suggest that the TB-*mas2*′ gene could have been selected in some tobacco populations by nature or by tobacco growers, as a result of changes in the root metabolism of these plants (Chen et al., 2016). The inverted repeat of TC partially aligns with TL from *A. rhizogenes* strain A4 (Chen et al., 2017).

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Some strains of agrobacteria carry two different T-DNAs on their Ti/Ri-plasmid (TL and TR regions) and can introduce separately or combined as a single insert. Potentially, this leads to a large variety of cT-DNA structures. However, most natural transgenic plants carry a single insert consisting of a partially inverted cT-DNA repeat. There is no proven hypothesis of why this is the case, although the following factors might be considered (Chen et al., 2017):

a) cT-DNA inserts in multiple sites will segregate during sexual propagation, favoring single inserts;
b) repeat structures are more tolerant to mutations, thus facilitating the preservation of important genes;
c) T-DNA transfer starts at the right border and proceeds to the left, therefore incomplete T-DNA structures will tend to have intact right borders and break off on the left.

After stable integration, cT-DNAs will evolve through point mutations, insertions, and deletions, in the same way as normal plant DNA (Chen et al., 2017). Many cT-DNA genes in natural transgenic plants are interrupted by stop codons or are partially deleted. Moreover, some genes of cT-DNA may become active. For example, *NgrolB* of *N. glauca* is inactive but can convert to an active form by the removal of two stop codons (Aoki, 2004). However, it is not clear whether the active form corresponds to the original *rolB* gene.

Other famous examples include *Linaria vulgaris* (common toadflax) and *Ipomoea batatas* (sweet potato). *L. vulgaris* cT-DNA is an exception to the usual rule of cT-DNAs: most of them have inverted repeats, while *Lv*T-DNA has direct repeats. *L. vulgaris* was shown to contain several sequences, homologous to T-DNA genes of mikimopine synthase (*mis*), *rolB*, *rolC*, *ORF13,* and *ORF14* (Kovacova et al., 2014; Matveeva et al., 2012). It is believed that the presence of cT-DNA in *Ipomoea* and other species may influence the special regeneration abilities of these plants: *Linaria* carries buds on its roots, which may greatly facilitate plant regeneration from hairy roots. *L. vulgaris* (but not *L. maroccana*) internode fragments easily form shoots and calli *in vitro*, even on a hormone-free medium (Matveeva et al., 2012).

In *I. batatas*, two sets of T-DNA were found: *Ib*T-DNA1 and *Ib*T-DNA2 (Kyndt et al., 2015). *Ib*T-DNA1 was found to contain four ORFs homologous to the tryptophan-2-monooxygenase (*iaaM*), indole-3-acetamide hydrolase (*iaaH*), C-protein (*C-prot*), and agrocinopine synthase (*Acs*) genes of *Agrobacterium* spp. *Ib*T-DNA2 contained at least five ORFs with significant homology to the *ORF14*, *ORF17n*, *rolB/rolC*, *ORF13*, and *ORF18/ORF17n* genes of *A. rhizogenes*. Such findings corroborated that sweet potato is naturally transgenic while being a widely and traditionally consumed food crop. It is believed that thanks to the constant advances in molecular biology and the popularity of sequencing, more plants of other genera will be found to carry some regions of agrobacterial T-DNA in nature (Aubin et al., 2021).

Agrobacterium strains have also been found as endophytes in many symptomless monocot species (Kang et al., 2020), which are known to be resistant to agrobacterial infection, and therefore hard to be transformed. The study of endophytic variation and how these endophytic strains differ from pathogenic strains is interesting. For this, endophyte isolates from young wheat and barley plants resistant to diseases were screened. Obtained isolates were studied and their plasmids were determined: two isolates with 200-kbp Ri-plasmids carrying *rol* genes and five isolates with 500-kbp Ti-plasmids were found. Moreover, both isolates with Ri-plasmids were examined on their ability to cause infection in *Nicotiana*. The experiment was successful, and the hairy root proliferation took place. Data strongly suggest in favor of the pathogen-reservoir plant hypothesis, i.e. that healthy wheat and other monocot plants are reservoirs for pathogenic strains of *Agrobacterium* (Fig. 2), which may aid in horizontal gene transfer among different plants.

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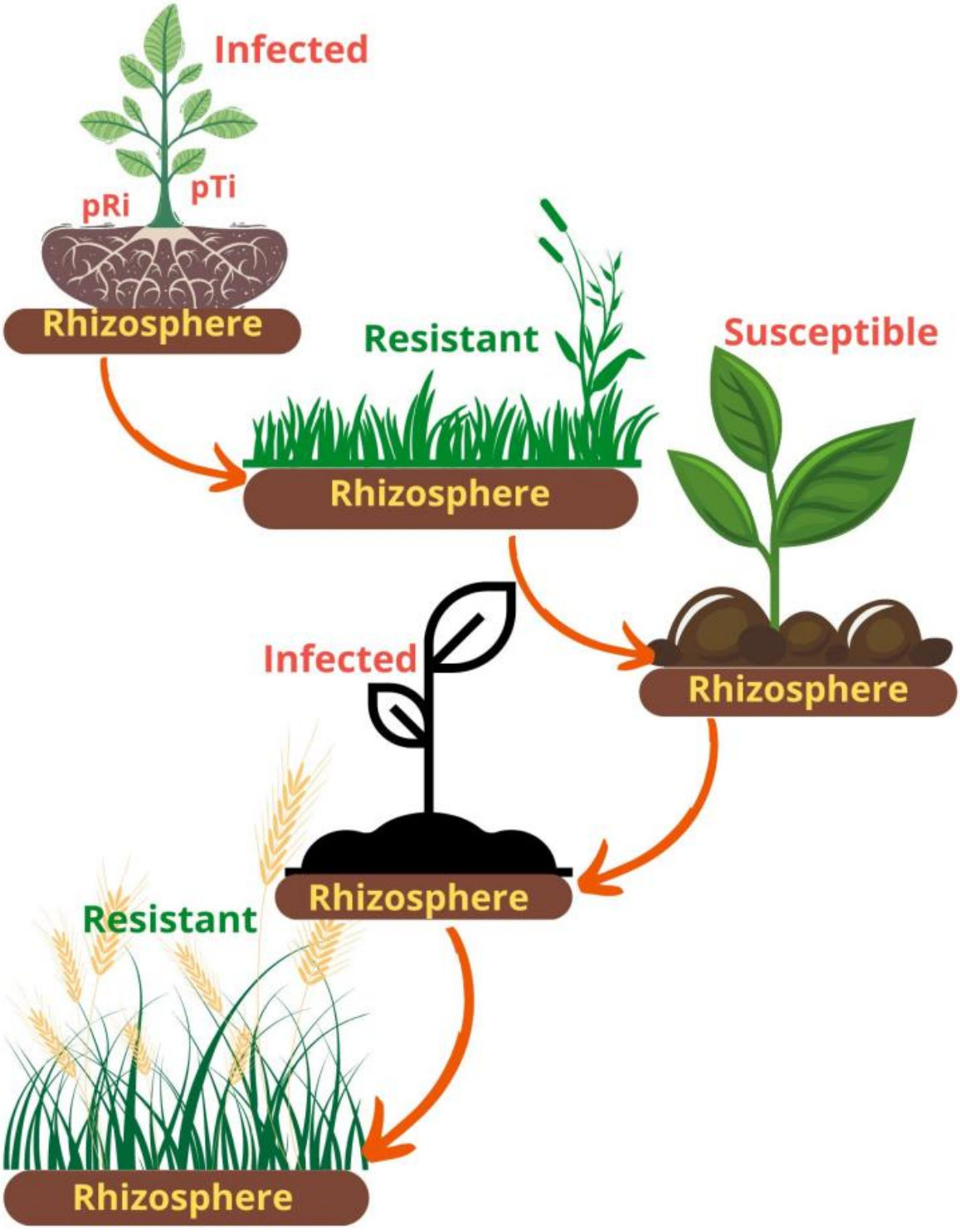

Figure 2 – The agrobacteria cycle as pathogens, rhizosphere inhabitants, and endophytes among susceptible and resistant plants (pathogen-reservoir plant hypothesis) (Kang et al., 2020)

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1.6. Plant-*Agrobacterium* interaction in nature

Agrobacteria are not just pathogenic bacteria that persist in plants, changing plant metabolism in bacteria's favor. They are soil bacteria, living in the rhizosphere, often found in soils of various origins, and appear to be among the most common inhabitants of these environments (Dessaux & Faure, 2018). Agrobacteria can live without harming the plant, as T-DNA incorporation occurs only in some wounded plants during various stages of their life (via wounds caused by growth, germination, subterranean insects, or mechanical injuries, such as pruning, grafting, and replanting of trees in nurseries (Garrity, 2005)). This means that most of the agrobacteria isolated from soils should be avirulent. This hypothesis was confirmed by many researchers from the early studies of agrobacteria in the 1970-1980s.

Indeed, pathogenic agrobacteria were very rarely found in soils and on roots, unless the environments were contaminated by infected plants, whereas nonpathogenic agrobacteria were commonly present with concentrations ranging from 10^3 to 10^7 CFU/g (Schroth et al., 1971; Bouzar & Moore, 1987; Burr et al., 1987). I.e., they did not harbor a Ti-plasmid, the key replicon that determines virulence, unless the soil had a history of crown gall or hairy root disease.

Such findings may suggest interesting features of agrobacteria that may allow them to colonize the highly competitive soil environment (Fig. 3, *A*). Indeed, it was determined that

a) agrobacteria may survive for weeks and months under oligotrophic conditions, including pure water (Iacobellis and Devay, 1986);
b) surface waters and aerosols contribute to the dissemination of *Agrobacterium* populations;
c) members of this genus are also resistant to osmotic stress, both by taking up osmoprotectants (Boncompagni et al., 1999) or by synthesizing them (Smith et al., 1990);
d) agrobacteria can survive for months in humic acids that are released into the soil from decomposed plants (Süle, 1978);
e) these bacteria evolved a wide metabolic capability to feed on rhizodeposits – root cell debris and exudates that are released from plant root system (Dessaux & Faure, 2018).

Agrobacteria are well equipped not only to survive in various soils but to compete against other bacteria (Fig. 3, *B*), living in that soil and taking the much-needed nutritious compounds from it. Indeed, agrobacteria have a set of potent siderophores, such as agrobactin (Ong et al., 1979) and hydroxamate (Penyalver et al., 2001), which permit an efficient recovery of iron in iron-deprived environments. In addition, agrobacteria are partly resistant to some antibiotics (listed earlier) and they can express a type VI secretion system (Ryu, 2015) that drives the injection of at least three effectors with enzymatic activities (DNase and putative peptidoglycan amidase) into neighboring competing bacteria (Ma et al., 2014).

Another set of adaptations helps agrobacteria withstand plants as well (Fig. 3, *C*). Plants can exude harmful to other bacteria metabolites, such as phenolic compounds, which have inhibition properties for fungi and bacteria. It is one of the possible systems of plant protection in case of wounding or stress. Despite that, agrobacteria possess an efflux pump active on some phenolic substances, such as medicarpin, coumestrol, ferulic acid, vanillyl alcohol, vanillin, coniferyl alcohol, coniferyl aldehyde, sinapyl alcohol, sinapinaldehyde, and syringaldehyde (Brencic et al., 2004; Baude et al., 2016).

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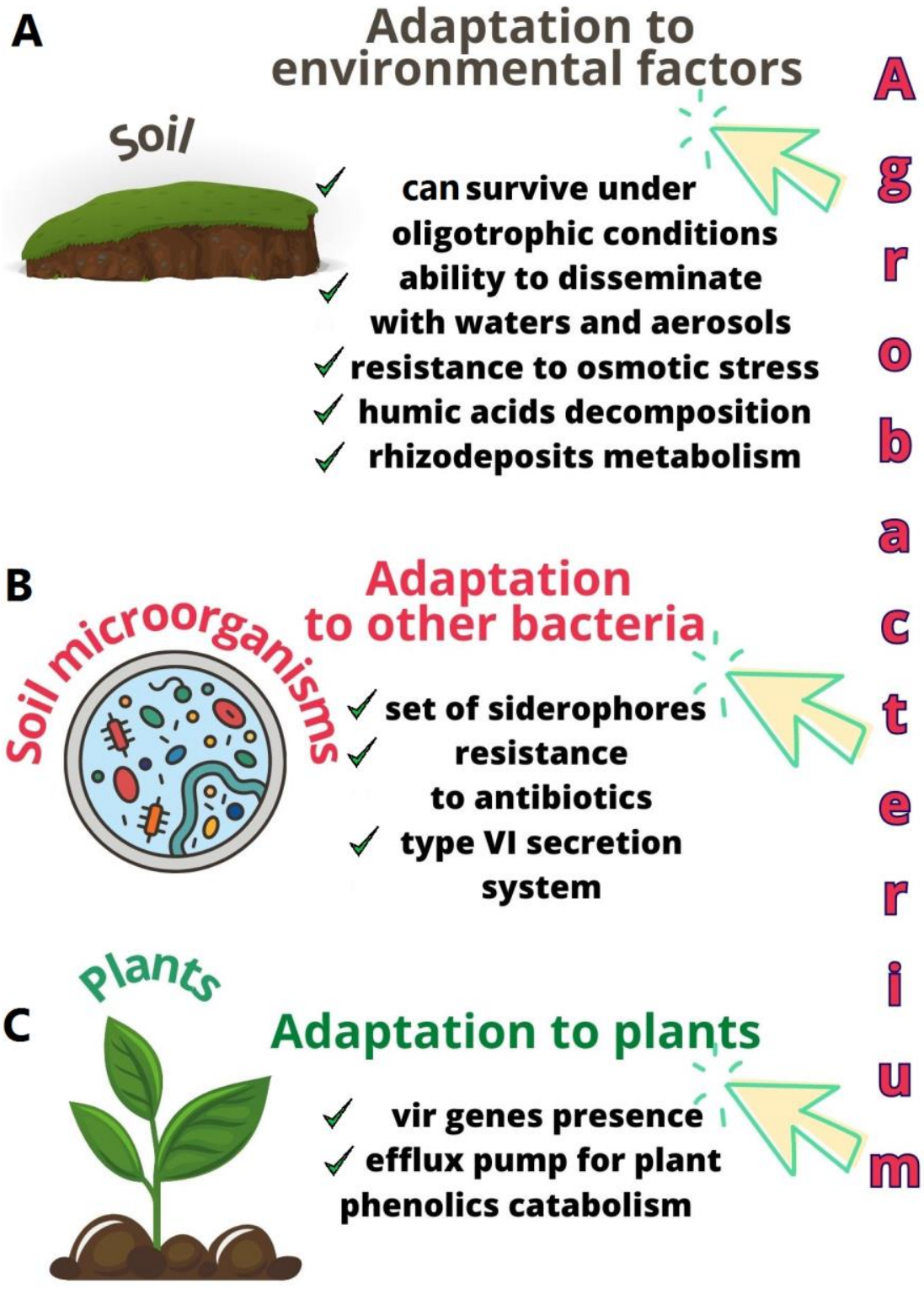

Figure 3 – Mechanisms of *Agrobacterium* spp. survival in soil: **A** – adaptations against environmental factors; **B** – adaptations against other bacteria; **C** – adaptations against plants

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In addition, pathogenic agrobacteria can detoxify other phenolics via the products of two Ti-plasmid genes, *virH1,* and *virH2*, located in the virulence region. These protein products share sequence homology with cytochrome P450-like enzymes (Kanemoto et al., 1989), and VirH2 is an O-demethylase active on over 15 phenolic substrates such as sinapinic acid and acetosyringone. Taken together, these data conclude that pathogenic agrobacteria are more resistant to phenolics than nonpathogenic ones, a result confirmed by the analysis of a *virH2* mutant (Brencic et al., 2004).

Remarkably, many of the above-mentioned phenolics (e.g., acetosyringone) are inducers of the *vir* genes of *Agrobacterium* and a few may also be chemoattractants (Parke et al., 1987). Indeed this wonderful adaptation allows agrobacteria to move upward in the concentration gradient toward the wounded plant cells. All these studies show that agrobacteria can thrive in soil and continue to be non-pathogenic. However, it is much more favorable for these bacteria to infect the plant and change its metabolism to feed on opines (as discussed earlier) and fill this "niche". In other words, it is better to shift from a generalist behavior in the soil and the rhizosphere to a specialist behavior in the tumor or hairy roots where they escape most microbial competitors and became a part of plant defense.

All in all, plant-*Agrobacterium* interaction in nature can occur in four different ways:

1) agrobacteria are living in the soil, feeding on rhizodeposits from plants and humic acids from decomposed plants, as well as other carbon and nitrogen sources from soils (Fig. 4, *A*);

2) *A. rhizogenes* and *A. tumefaciens* infect the wounded plants and can incorporate their T-DNA from Ri-/Ti-plasmids into the plant genome (in the case of dicots and some gymnosperms), changing plant metabolism to feed on produced opines (Fig. 4, *B*);

3) plants in nature carry some regions of agrobacterial DNA in the form of incorporated cT-DNA, when agroinfection took place in ancient times and deactivation of incorporated *rol* genes and other ORFs occurred (Fig. 4, *C*);

4) agrobacteria persist in plants (mostly monocots) without causing disease and can transfer *tzs* genes of phytohormone *trans*-zeatin synthesis (Han et al., 2013; Hwang et al., 2013), which could enhance plant growth and, in return, would favor the endophytes to live within these plants (Kang et al., 2020; Hooykaas & Hooykaas, 2021) that may be seen as some form of mutually beneficial coexistence between agrobacteria and plants that are not susceptible to agrobacterial infection in nature (Fig. 4, *D*).

1.7. Effect of *rol* genes

T-DNA of Ri-plasmid of *A. rhizogenes* type strain A4 contains two regions: TL-DNA and TR-DNA. The first has four *rol* genes ("rooting locus"): A, B, C, and D, which improve plant cell susceptibility to auxins and cytokinins and are responsible for the formation of these roots (Alcalde Cho et al., 2022; Mauro et al., 2017; Bulgakov et al., 2018). TR-DNA contains genes related to auxin biosynthesis, known as *aux*1 and *aux*2. Both regions can be transferred to the nuclear genome of infected plant cells independently (Nemoto et al., 2009).

All T-DNA genes and ORFs may be subdivided into 3 groups: the ones that encode opine synthesis, the ones that encode hormone synthesis, and plast genes. The latter includes *rol* genes and some ORFs of Ri-plasmid that can change the development of plants in various remarkable ways. The name "plast" originates from the "developmental plasticity" function of these genes.

The *rol* genes were initially defined based on the capacity of *A. rhizogenes* A4 T-DNA mutants to induce hairy roots on *Kalanchoe daigremontiana* leaves (White et al., 1985). The study showed that *rolB* plays a key role in the hairy root phenomenon, as *rolB* mutation

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abolished hairy root growth. *rolC* mutation showed that this gene is not essential for root induction on *K. daigremontiana* leaves, but in its absence, root growth was retarded. It was also shown that the *rolABC* combination is sufficient to induce the typical hairy root phenotype. Moreover, A4-*rolB* induces roots by itself (although these differ from *rolABC* roots), as well as *rolC* (only under 35S promotor though). However, one should keep in mind that the characteristics of obtained hairy roots are significantly dependent on the species of transformed plant, agrobacterial strain (see Sathasivam et al., 2022), and culture conditions.

For example, one of experiments showed that apple rootstock M.9/29 *rolB* tissues were rooted efficiently on a hormone-free medium (contrary to untransformed rootstocks). However, quite unexpectedly, root growth, length, and morphology of the regenerated plants were the same as for untransformed apples (Zhu et al., 2001). It was also determined (Offringa et al., 1986) that *Agrobacterium* strains with an agropine-type Ri-plasmid may not only cause hairy root induction but can also induce tumors in some plant species. *Agrobacterium* strains containing the TR-region but not the TL-region of the Ri-plasmid were still tumorigenic (those carrying *aux* genes) on certain plant species but were no longer capable of hairy root production. However, later research (Mauro et al., 2017) discovered that the knock-out of the *rolB* gene causes the plasmid to be avirulent.

Now, let us consider the mechanisms of *rol* genes in more detail.

The mechanism of *rolA* (*orf10*) action. The *rolA* was found on all Ri-plasmids. It encodes a small protein that is suggested to be involved in the metabolism of gibberellins and other classes of hormones, as *rolA* incorporation decreases their content and can cause dwarfism in some plants (Dehio et al., 1993; Pistelli et al., 2010; Ozyigit et al., 2013). It was also reported that the *rolA* gene is responsible for changes in polyamines metabolism (Martin-Tanguy et al., 1996).

The mechanism of *rolB* (*orf11*) action. The function of the *rolB* gene is the mechanism of "emergency" root formation in plants. In addition, it affects auxin metabolism and auxin perception (Otten, 2018). Thus, it plays an essential role in the early stages of hairy root induction (Dilshad et al., 2021). Adventitious roots induced by the *rolB* gene produce large lateral roots in tissue culture. These phenomena indicate that the *rolB* protein has a crucial effect on the formation of roots. Therefore, elucidation of the function of the *rolB* protein is necessary for understanding of root formation in plants (Pistelli et al., 2010). The difference in the growth rate among hairyroot lines is due to the difference in the expression level of the *rolB* gene (Tanaka et al., 2001). A proper level of *rolB* expression appears necessary for the active growth of hairy roots because either a high or a low level correlates with impaired growth. Overexpression of the *rolB* gene under the control of the CAMV35S RNA promoter (P35S) suppresses adventitious root induction (Spena et al., 1987) and leads to cell death (necrosis) both in callus and in leaves of young plants (Schmulling et al., 1988). It was suggested that rolB protein is a beta-glucosidase, tyrosine phosphatase, or an auxin-binding protein. However, such hypotheses were withdrawn (Mauriel et al., 1991; Nilsson et al., 1993). *rolB* gene is also considered the gene that mostly influences plant secondary metabolism and activates plant defense reactions out of all *rol* genes (Bulgakov et al., 2002; Kiselev et al., 2007; Pistelli et al., 2010; Dilshad et al., 2021). Its expression increases tolerance to biotic and abiotic stresses (Veremeichik et al., 2012; Bulgakov et al., 2013; Arshad et al., 2014) and enhances resistance against fungi (Arshad et al., 2014). Additionally, the *rolB* gene showed to be involved in RNA silencing pathways through microRNA overexpression (Bulgakov et al., 2015). Finally, the *rolB* gene is involved in the activation of the transcription factors of most specialized metabolites in hairyroots and the expression of chaperone-type proteins (Bulgakov et al., 2018). It is also considered that *orf13* acts synergistically with *rolB* and may replace auxin required for hairyroot induction, suggesting it has auxin-like activity (Hansen et al., 1993; Aoki and Syono, 1999).

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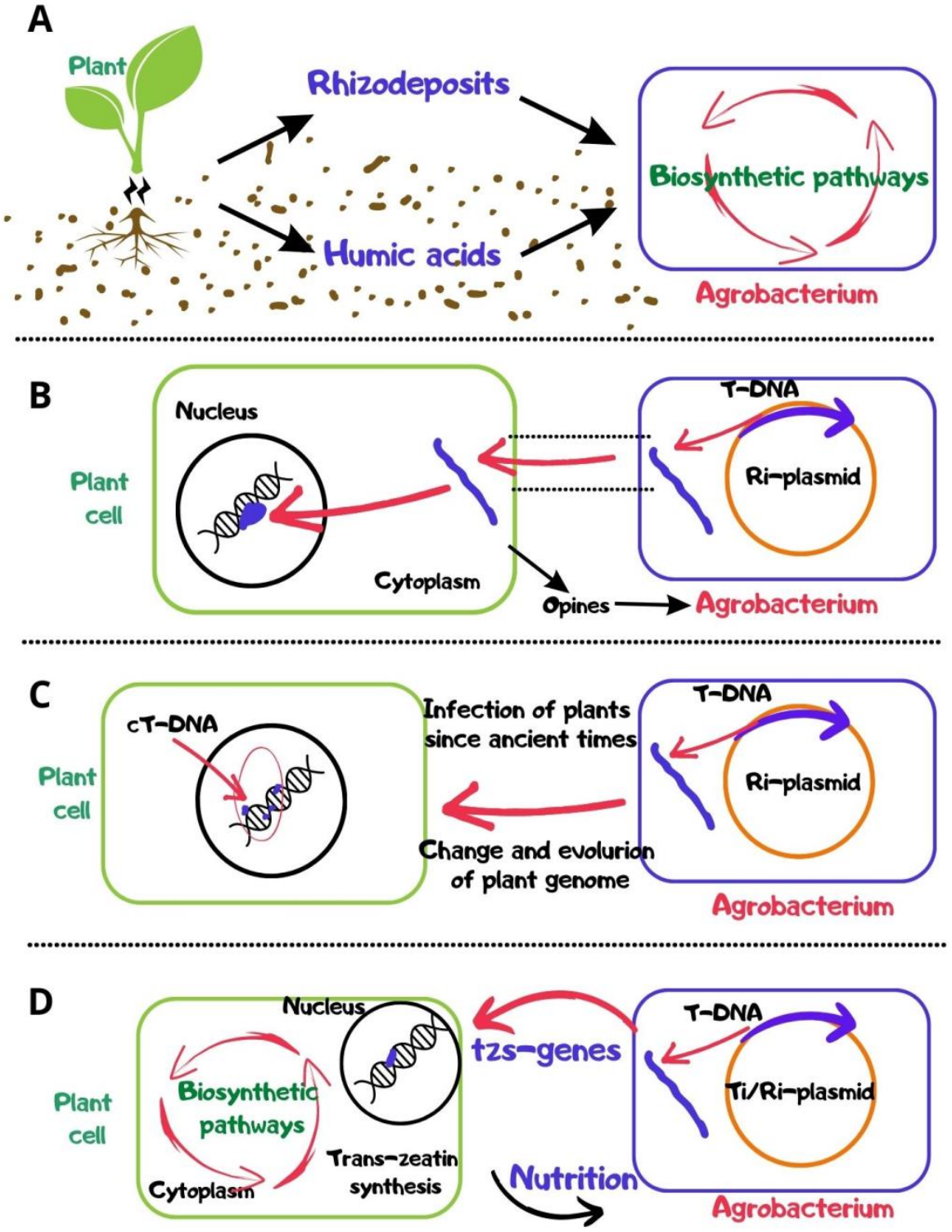

Figure 4 – Plant-*Agrobacterium* interaction in nature: **A** – agrobacteria as free-living organisms in soil; **B** – agrobacteria as the causative agent of hairy root and crown gall diseases; **C** – agrobacteria as one of the agents in horizontal genes transfer in the form of cT-DNA; **D** – possible mutually beneficial coexistence between agrobacteria and some plants

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The mechanism of *rolC* (*orf12*) action. *rol*C is considered the most conserved of all *rol* genes and has a minor impact on root formation (Makhzoum et al., 2013). The effects of the *rolC* gene are well documented in many plant species, as they produce essentially similar phenotypes. This gene affects plant size and structure, including reduced height, internode length, fertility, apical dominance, and increased number of flowers (Smith et al., 2006). Other morphological effects are changes in the size, color, and shape of leaves (Pistelli et al., 2010). The different degrees of dwarfness due to the *rolC* effect have been observed among independent transformants carrying the same *rolC* gene construct. These differences depend on several factors as the site of integration, copy number, mutation, somaclonal variation, and changes in expression level (Kiyokawa et al., 1996; Giovannini et al., 1997; Pistelli et al., 2010). The effects on plant morphology of the *rolC* gene may be due to cytokinin-beta-glucosidase activity that increases cytokinin levels (Estruch et al., 1991). It was demonstrated that *rolC* has a minor stimulatory effect on secondary metabolism as well, influencing the levels of tropane alkaloids, pyridine alkaloids, indole alkaloids, ginsenosides, and shikimate-derived anthraquinone phytoalexins, without changing general plant defense pathways (Bulgakov et al., 2003; Palazon et al., 2003; Cardillo et al., 2013; Hashemi and Naghavi, 2016). Interestingly, *rolC* is induced by sucrose (Nilsson et al., 1996) and it can counteract the necrotic effects of the *rolB* gene (Röder et al., 1994). Nevertheless, the mechanism for this antagonistic activity remains unexplained. It may be due to the possible function of *rolB* in increasing or decreasing reactive oxygen species (ROS) signaling, while *rolC* acts as a suppressor of ROS (Paolis et al., 2019).

The mechanism of *rolD* (*orf15*) action. It is considered that *rolD* stimulates reproductive phase transition in plants, enhanced flowering (through changes in the concentration of plant hormones), minorly stimulates root growth, and influences plant performance and the defense response to pathogens (Mauro et al., 1996). Biochemical assays have shown that *rolD* encodes an ornithine cyclodeaminase able to catalyze the NAD^+-dependent conversion of ornithine to proline, thus inducing acceleration and stimulation of flowering in both plants and tissue cultures (Pistelli et al., 2010). Thus, the role of *rolD* in hairyroot formation is marginal, but the gene can influence plant development through its metabolic activity (Trovato et al., 2018). This gene shares about 55% identity with adjacent *orf16* (Hooykaas & Hooykaas, 2021).

Most studies on the expression of *rol* and *aux* genes in hairy root cultures have focused on demonstrating their effect on secondary metabolism and morphology of transformed roots and regenerated plants. Alcalde Cho et al. (2022) focused on analyzing the relationship between *rol* and *aux* genes expression and the hormonal profile, which is a determinant in root development and morphology using machine learning models. They studied 10 hairy root lines of *Centella asiatica* that had noticeable differences in branching rate, growth rate, and biomass accumulation.

The scientists selected *rolA*, *rolB,* and *rolC* genes, as they have been shown to play the most relevant role in hairy root development (Sarkar et al., 2018; Bahramnejad et al., 2019), and the *aux1* gene. In their study, the expression level of all *rol* genes was higher than that of the *aux* gene, which may be since only the presence of TL-DNA genes is required for long-term hairy root growth (Chriqui et al., 1996).

The results showed that the expression of genes varies widely among the lines: lines L3, L4, L6, and L7 had the lowest expression of all genes; L10, L12, and L14 had high expressions of the *rolC* and *rolB* genes; L1, L2, and L8 showed higher expressions of the *rolA* and *aux1* genes. A positive correlation was observed between centelloside content, branching, biomass productivity, *rol,* and *aux*1 genes. The highest centelloside productions were strongly related to *rolA*, *rolB,* and *aux1* genes.

The cluster analysis of hormones based on the influence of gene expression was performed as well. Abscisic acid (ABA) was most influenced by the expression of *rolB*,

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followed by *rolA* and *rolC*. Isopentenyl adenosine (IPA) was heavily influenced by *aux*1, followed by *rolA*. 2-Isopentenyl adenine (2iP) was mostly affected by *rolC*. Gibberellins (GA_4) synthesis was slightly affected by *rolA* and *aux1*. Indole 3-acetic acid (IAA) was influenced by *rolA* and *rolC*. Salicylic acid (SA) was affected by *rolB*. Other plant hormones (jasmonates and *trans*-zeatin) were not greatly influenced by *rol* or *aux1* gene expression.

In addition to the studies of Ri-plasmid T-DNA genes' functioning as hairy root inducers, many researchers were interested in the morphological differences of those transformed roots from the wild-type roots on a microscopic level. Data from various articles (Lincoln et al., 2002; Ho-Plágaro et al., 2018; Daspute et al., 2019; Jian et al., 2009; Ishida et al., 2011; Marsh et al., 2014; Plasencia et al., 2016) is in favor of the ability of hairy roots to retain the structure and fine morphology of wild-type roots of the same species. This information is crucial, as it shows that hairy roots can be used as a potent system for the profound study of the function of genes that play a role in the development of the root structures in nature.

Let us look at some of the examples.

Jian et al. (2009) successfully transformed *Lotus corniculatus* via *A. rhizogenes* infection. They identified hairy roots with the help of GUS staining and GFP detection. The microscopy and PCR analysis proved the transgenic plants carried both GFP and GUS genes. Additionally, the comparison of hairy roots and non-transformed roots showed their similarity in structure. To further verify gene transfer, GFP and GUS expression were monitored on the whole plant level: scientists obtained regenerated plants from hairy roots and conducted GUS staining and GFP detection as well. The transformation events were additionally confirmed by Western blot using an anti-GFP antibody.

Ishida et al. (2011) were interested in whether the possibility of parasitic roots retaining their specialized structures will be changed or even canceled after their transformation. The scientists successfully obtained hairy roots of *Phtheirospermum japonicum* from cotyledons (using *A. rhizogenes* and acetosyringone treatment) and verified their transgenicity by genomic PCR, Southern blot, and RT-PCR methods.

This plant forms such specialized structures – haustoria – through which *P. japonicum* obtains water and nutrients directly from host plants. To determine whether or not transgenic roots can form haustoria, transgenic roots were placed in a medium containing 10 μM DMBQ for 2 days, as DMBQ (2,6-dimethoxy-1,4-benzoquinone) is a haustorium-inducing chemical. Competent haustoria were formed and studied using bright field and fluorescence microscopy. They were morphologically indistinguishable from those formed on non-transgenic roots. To test if transgenic *P. japonicum* roots can infest host plants, they were co-incubated with rice and maize. In both cases, haustoria developed and attached to the hosts as well as the wild type. Thus, the morphologically normal development of haustoria in *P. japonicum* hairy roots indicates that the hormonal effects caused by the insertion of *rol* genes (Offringa et al., 1986) do not influence the parasitic competence of the transformed roots.

Marsh et al. (2014) established hairy roots of *Scutellaria lateriflora* from internode sections and cultivated them in a liquid medium. The microscope study of transgenic roots exhibited a high degree of branching and the abundancy of very fine single-cell root hairs (long unicellular tubes and short papillae), which are the typical characteristics of *A. rhizogenes*-mediated transformed roots reported for many genera (Flores and Medina-Bolivar, 1995).

Hairy root cultures do not need supplementation with hormones to grow. However, sometimes auxins are added to obtain more root biomass in less time. Indole-3-butyric acid (0.5 mg/l) was added in this case. Agropine type *A. rhizogenes* strains, such as ATCC 15834 used in this study contain two T-DNAs, which are important for the establishment of hairy root cultures. The TL-DNA contains the *rol* genes and is considered vital for the hairy root

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initiation, while the TR-DNA contains the *aux* genes necessary for auxin biosynthesis. The scientists performed PCR analysis to confirm the incorporation of both *rol* and *aux* genes. These genes were transferred successfully, thus showing that the absence of vigorous growth of hairy roots in a liquid medium is not caused by the loss of *aux* genes. Possible explanations could be due to the positional genome integration effect of the T-DNA or an alternative functionality of the *rol* and *aux* genes in *S. lateriflora*, as the place of transferred genes (using agroinfection methods) is always undetermined.

Plasencia et al. (2016) developed hairy root cultures of *Eucalyptus grandis* as a model system of rapid *in vivo* analysis of transgenes. They showed that *Eucalyptus* hairy roots are suitable for medium-throughput functional characterization of genes enabling, among others, protein subcellular localization, spatial and temporal patterns of gene expression, and down-regulation of endogenous genes. To evaluate whether transgenic hairy roots could be used as a system to explore the function of genes involved in xylem secondary cell wall formation, the scientists compared the radial patterning and xylem anatomy of hairy roots relative to wild-type roots. The observations under light microscopy and UV light (exciting the natural lignin autofluorescence) showed that both primary and secondary xylem of transformed roots developed similarly to nontransformed ones. Thus, transferred *rol* genes did not influence the morphological structure of the roots.

Therefore, the years-long study of *rol* genes is not finished and is carried on both for a better understanding of the function of genes in hairy root cultures and for obtaining new features suitable for various usages.

1.8. Transfer of foreign genes

In previous sub-chapters, we revised *A. rhizogenes* as a soil inhabitant that, at the same time, is a causative agent of hairy roots. We recalled the structure of Ri-plasmids and T-DNA that is transferred into the plant cell nuclei, and we mentioned the effect of *rol*-genes as secondary metabolism inducers. All the abovementioned information supports *A. rhizogenes*-mediated transformation as a powerful and potent for plant metabolism change in favor of producing new substances. Now, let us get acquainted with the different agrobacterial vectors that may be used.

The simplest vector that can be used for the plant transformation to obtain hairy roots with boosted secondary metabolites synthesis is the Ri-plasmid of the wild strain, such as A4. As it was mentioned earlier, it harbors two regions of T-DNA (TL- and TR-DNA), *vir* genes, and genes for opine catabolism. This strain may be used for the induction of new hairy root lines with different sets and content of metabolites, e.g. phenolic substances, and even new metabolites that were not detected in the control plant.

However, *A. tumefaciens*-mediated transformation is commonly used as well. Its main difference is that wild type *A. tumefaciens* strains are not used for genetic engineering nowadays. The cause is that wild type *A. tumefaciens* carry tumor-inducing, i.e. crown gall inducing genes in its T-DNA (that encode auxins and cytokinins production). Moreover, these genes do not boost secondary metabolism as *rol*-genes do. That is why, from early on, scholars decided to "cut out" all the genes from the T-DNA of *A. tumefaciens* (between LB and RB) and substitute them with the genes of interest. These early studies include the work of Zambryski et al. (1983), in which the oncogenic sequences between LB and RB of *A. tumefaciens* were replaced by pBR322 – plasmid of *E. coli*. The resulting plasmid pGV3850 still mediatedthe efficient transfer of T-DNA, which confirmed that even minimal T-DNA without oncogenes is enough to be incorporated into the plant genome.

Ti-plasmids that do not carry oncogenic genes are called "disarmed" plasmids and *cis*-plasmids (as all needed genes for plant transformation were on the same

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plasmid/vector). They become widely used and gave rise to the origin of new vectors for plant transformation.

Apart T-DNAs, Ri- and Ti-plasmids have a high degree of homology (Fig. 5). Activation, processing, and movement of the T-DNA from the bacteria to the plant cell are highly sustained by *vir* genes in both species (Ozyigit et al., 2013). The organization of *vir* genes in operons is highly similar. The only difference is that Ri-plasmids lack *virE1* and *virE2* genes, and at the same time, they carry *GALLS* genes of the same function (considered in the previous sub-chapters). The roles of *vir* genes include:

a) sensing plant phenolic compounds (e.g., acetosyringone);
b) induction of other *vir* genes and *chv* genes (chromosomal genes that take part in virulence) expression;
c) export of T-DNA into the cell;
d) promotion of T-strand synthesis;
e) topoisomerase and endonuclease functions.

Still, the obtained "disarmed" plasmids of *A. tumefaciens* were quite "bulky" for the quick design of new vectors with selective genes and genes of interest. Thus, scientists were interested if it is possible to separate the much-needed *vir* region from the T-DNA. Such separation was made (Hoekema et al., 1983; Bevan, 1984) and it confirmed the idea that *vir* genes act even in trans-position, i.e. they can transfer T-DNA while existing on a different plasmid. This originated a new type of vectors, trans-vectors or binary vectors (Fig. 6), which was also confirmed later in *A. rhizogenes* (Simpson et al., 1986).

Binary vectors include two plasmids/vectors: Ri-plasmid or "disarmed" Ti-plasmid, which is usually called T-binary vector, and helper plasmid, which is sometimes called *vir* helper plasmid. T-binary vector includes T-DNA and vector backbone. T-DNA has left and right border sequences (usually come from octopine or nopaline plasmids). Besides, it may carry reporter genes (GUS, LUC, GFP), selective genes (resistance to antibiotics or herbicides), and genes of interest with promoters and terminators. Typically, the vector backbone carries origins of replication for agrobacteria and *Escherichia coli* (both are needed for vector manipulation and maintenance) and some selectable markers. *E. coli* is used for plasmid manipulation, as it is a fast-growing bacterium. Thus, it helps to increase the plasmid yield. Helper plasmid carries *vir* region and origin of replication for agrobacteria. It can also carry another type of ori gene, *rep ABC*, with the same function. Sometimes "disarmed" Ti-plasmids without any incorporated T-DNA can serve as helper plasmids. Binary vectors can be "evolved" into superbinary vectors with the addition of extra *vir* genes to the T-binary vector. This region is called *S vir* and is used for the additional virulence of binary vectors. Such a technique may be useful for monocots and recalcitrant plants that are hard to transform. The last considered type of agrobacterial vectors is ternary vectors, introduced in 2018 by Anand et al. The idea is to use the third plasmid, called accessory plasmid or *vir* accessory plasmid, which carries an additional virulence gene cluster (Fig. 6). This plasmid has a structure similar to helper plasmid. Such a vector system is used for recalcitrant plants as well.

Now, let us review the general scheme of selecting an appropriate agrobacterial vector for plant transformation (Fig. 7). At first, you should define the object of the genetic transformation, i.e. study the plant you have chosen to transform. For a dicotyledonous plant that is easy to transform, wild-type *A. rhizogenes* or binary vector will be enough. However, if you choose to work with monocots or recalcitrant plants, you should use superbinary or ternary vectors. After that, you have to determine the goal of your study. If you want to initiate hairyroot cultures without any added genes, you should use wild-type *A. rhizogenes*. If you need to obtain lines with new characteristics (e.g., expressing recombinant proteins) you have to use binary, superbinary, or ternary vectors carrying the genes of interest. If you aim to conduct a functional study, you should use CRISPR/Cas

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vectors (see below) or vectors for suppressing plant genes by RNA interference.Then, you have to revise the publications on the plant and vector of choice, as it is much easier to use the already working system than to assemble a vector "from scratch" – with all new promoters, terminators, markers, and so on. The last step is the proceeding of transformation according to a protocol of choice, preferably with different vectors (or some agrobacterial strains).

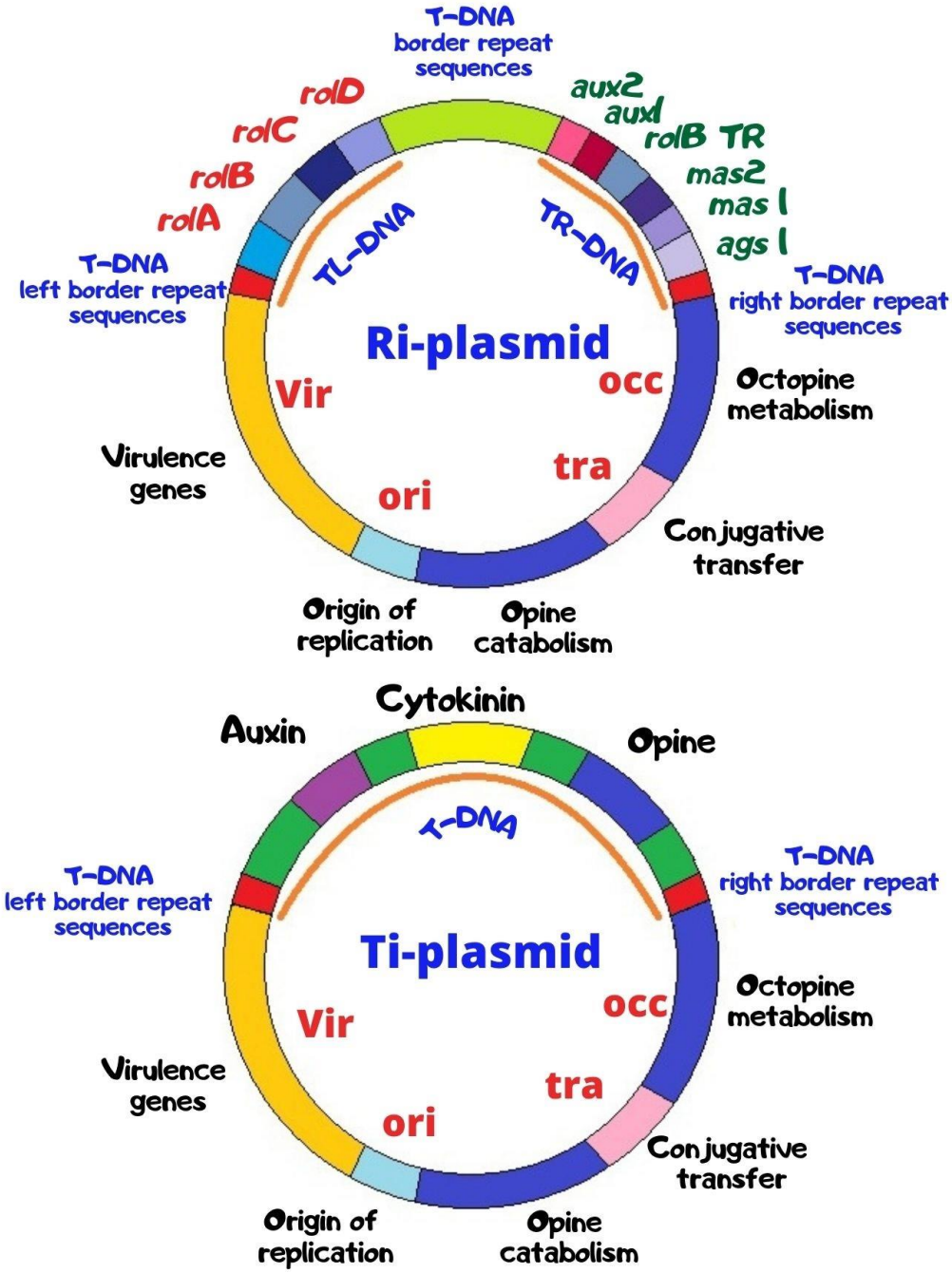

Figure 5 – Agropine type Ri-plasmid of *A. rhizogenes* and Ti-plasmid of *A. tumefaciens* (Mukherjee & Gantait, 2023)

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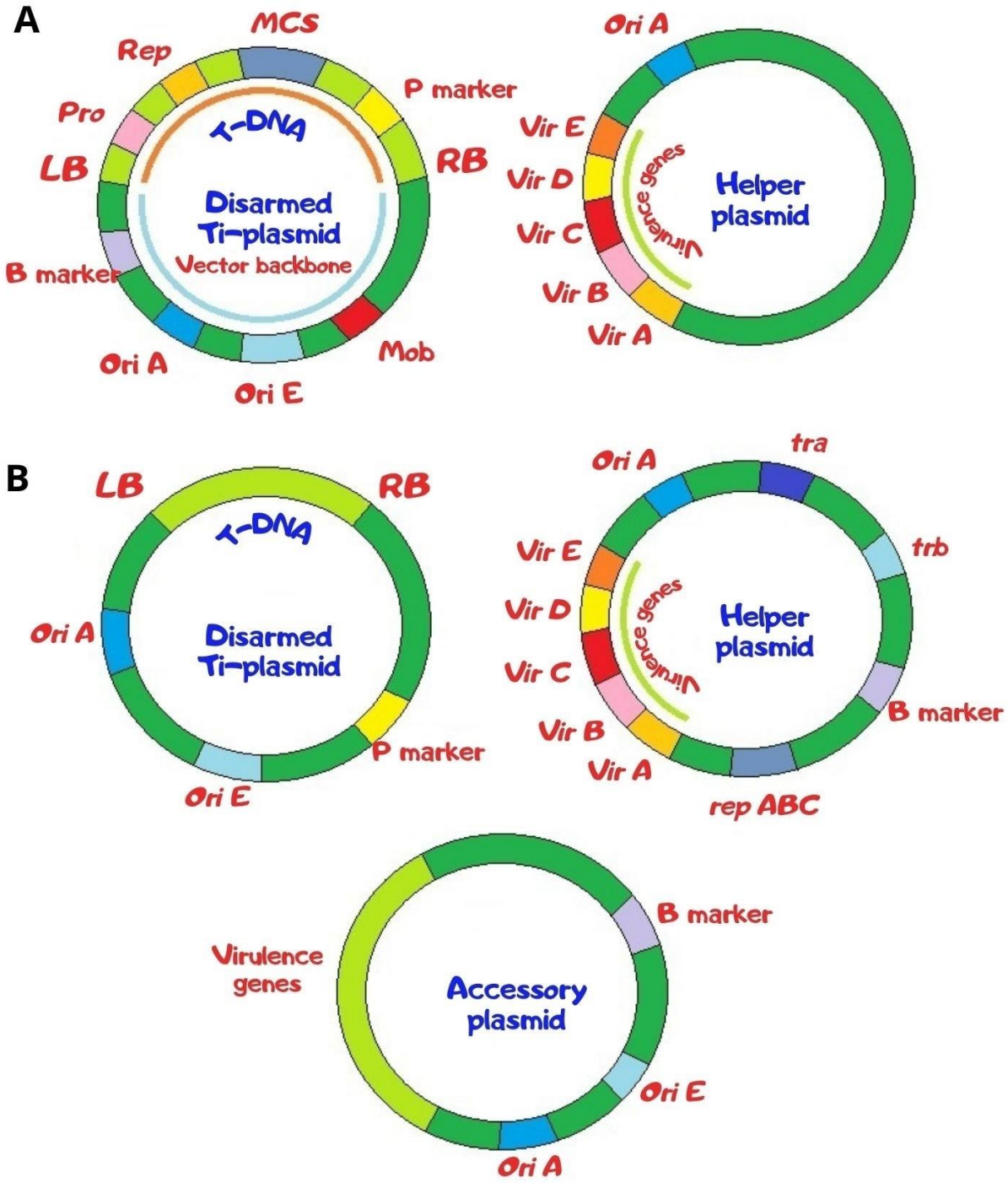

Figure 6 – Different agrobacterial vectors. **A** – Components of the binary vector: Disarmed Ti-plasmid and Helper plasmid (B marker – bacterial selectable marker, LB – Left border, Mob – Mobilization function, MSC – Multiple cloning sites, Ori A – origin of replication for *Agrobacterium*, Ori E – origin of replication for *E. coli*, P marker – Plant selectable marker, Pro – Promoter, RB – Right border, Rep – Reporter gene). **B** – Components of the ternary vector (Tra/trb – transcriptional activators, rep ABC – ABC replication origin) (Gallego, 2023)

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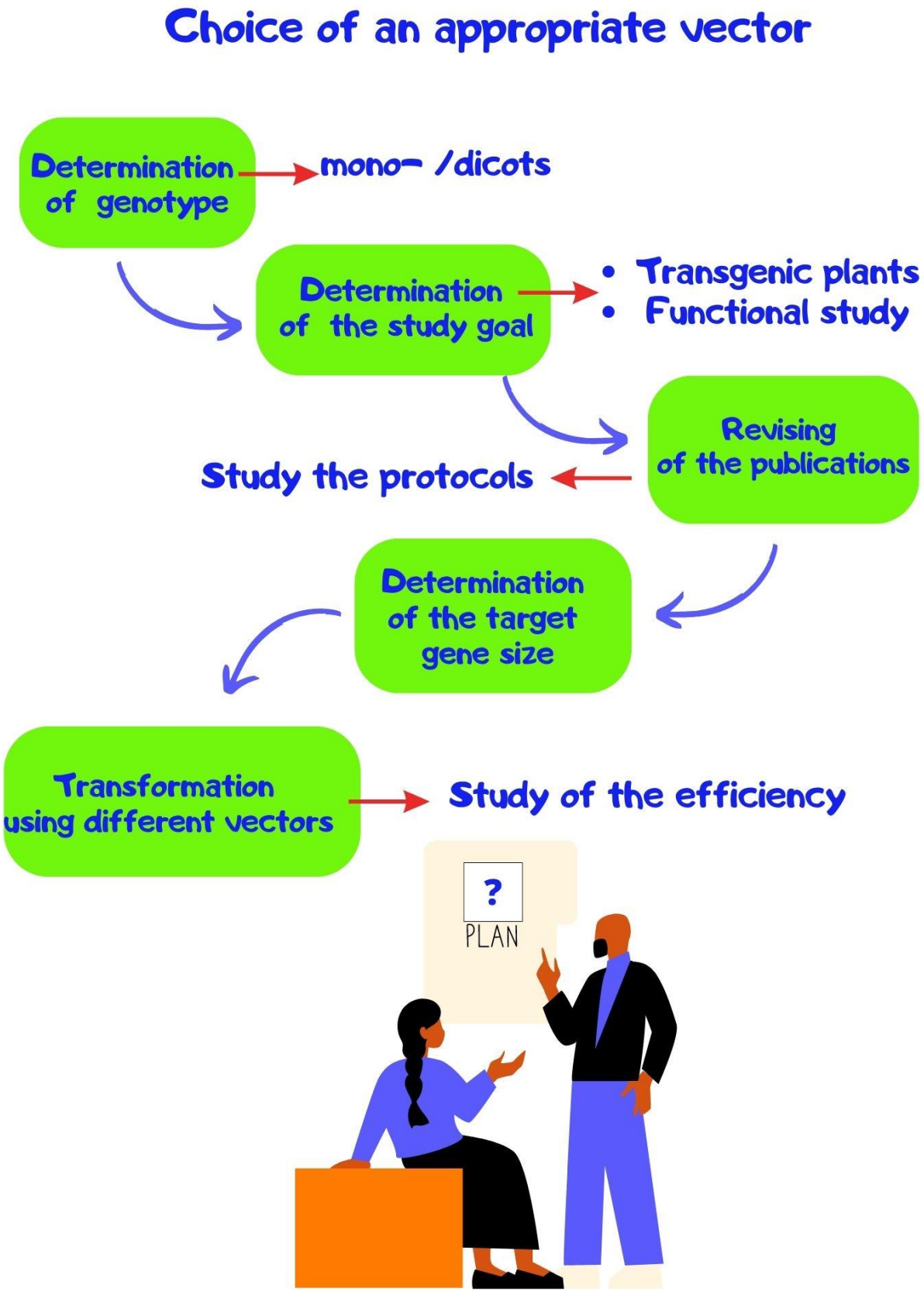

Figure 7 – The general scheme of selecting an appropriate agrobacterial vector for plant transformation (Gallego, 2023)

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Table 1 – The results of studies on the determination of various aspects of the antioxidant activity of transgenic roots, obtained via *A. rhizogenes*-mediated transformation without additional genes

Plant species	***A. rhizogenes* strain**	**Results**	**References**
Rehmannia glutinosa (Gaertn.) Steud.	A4	Decrease of TBARS (thiobarbituric acid reactive substances) level by 50% (shoot extract) and 30% (root extract)	(Piątczak et al., 2016)
Physalis ixocarpa Lam.	ATCC 15834	Increase of: TAC (total antioxidant capacity) by 39% (root extract) and 64% (leaf extract), the total content of phenols, ascorbate content in the leaves	(Bergier et al., 2012)
Artemisia vulgaris L.	A4	Increase in: the total content of flavonoids (from13.3 ± 1.0 to 73.1 ± 10.6 mg RE/g DW), antioxidant activity (EC_{50} = 0.23…0.36 mg DW compared to control 1.09 ± 0.05 mg DW	(Matvieieva et al., 2019a)
Lactuca serriola L.	AR15834	Increase in: the total content of phenols (by 54.8 … 96.7%), the total content of flavonoids (by 38.1…76.2%), total reducing power (by 56.7…96.7%), antioxidant activity (by 31.6…50%)	(El-Esawi et al., 2017)
Isatis tinctoria L.	LBA9402	Increase in: the total content of flavonoids (438.10 µg/gDW compared to control 341.73 µg/gDW), antioxidant activity (EC_{50} = 0.39…0.41 mg DW compared to the control 0.48…0.56 mg DW)	(Gai et al., 2015)
Astragalus membranaceus (Fisch.) Bunge	LBA9402	Increase in: the total content of flavonoids (234.77 µg/gDW compared to the control 187.38 µg/gDW), antioxidant activity (EC_{50} =1.40…1.73 mg/mL compared to the control 1.96…2.17 mg/mL)	(Jiao et al., 2014)

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Plant species	*A. rhizogenes* strain	Results	References
Polygonum multiflorum Thunb.	KCTC 2703	Increase in: the content of anthraquinones (emodin 211.32 μg/gDW and physcion 353.23 μg/gDW, which is 3.7 and 3.5 times more than in control), flavonols (804.0 μg/g compared to 549.5 μg/g in the control), hydroxycinnamates (833.5 μg/g compared to 599.5 μg/g), the total content of phenols (8175.30 μg/g compared to 5633.07 μg/g GAE), the total content of flavonoids (76.15 μg/g QE compared to (43.03 μg/g QE), antioxidant activity Decrease in: the content of hydroxybenzoic acid (1355.0 μg/g compared to 1481.5 μg/g), rutin	(Thiruvengadam et al., 2014)
Prosopis farcta (Banks & Sol.) J.F. Macbr.	LBA9404, AR15834 and A4	Increase in: the total content of flavonoids by 1.54 and 2.52 times compared to the control (non-transformed roots) and callus, respectively	(Zafari et al., 2018)
Althea officinalis L.	A4, A13, ATCC15834	Increase in: the total content of phenols (up to 1.57 ± 0.1 mg/g) and flavonoids (up to 3.47 ± 0.3 mg/g)	(Tavassol & Safipour Afshar, 2018)

Table 2 – The results of studies on the determination of various aspects of the antioxidant activity of transgenic plants, transformed with *A. tumefaciens*

Plant species	*A. tumefaciens* strain	Vectors/genes used	Results	References
Ipomoea batatas (L.) LAM. cultivar Xushu 29	*Agrobacterium tumefaciens* EHA105	pGWB5 vector with *IbTC* and *GFP*	Increased content of α-tocopherol (leaves: 188.9 ± 36.8 μgg-1 DW, 3.3 times more than control; roots: 33.5 ± 4.1 μgg-1 DW, only 10% more in 1 plant)	(Kim, 2019)
Nicotiana bentamiana Domin.	*Agrobacterium tumefaciens* GV3101	pGWB5 vector with *IbHPPD*, *IbHPT*, *IbMPBQMT*, *IbTC, IbTMT Ipomoea batatas*	Increased content of α-tocopherol in leaves: 1.81…2.84 times more than the control	(Ji et al., 2016)

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Plant species	*A. tumefaciens* strain	Vectors/genes used	Results	References
Perilla frutescens (L.) Britton	*Agrobacterium tumefaciens* LBA 4404	*γ-tmt* gene (pYBI121 vector) from *Arabidopsis thaliana*	The antioxidant potential of extracts increased 1.25…2.77 times	(Ghimire et al., 2015)
Malus domestica Borkh.	*Agrobacterium tumefaciens* EHA105	MdNAC1 vector with genes from *Arabidopsis thaliana*	Decrease in the content of free O_2^-radicals: 195…236 nmolg^{-1}FW compared to contro l412 nmolg^{-1}FW	(Jia et al., 2019)
Brassica juncea (L.) Czern.	*Agrobacterium tumefaciens* GV 3101	*γ-tmt* gene (pYBI121 vector) from *Arabidopsis thaliana*	SOD, CAT та APX (ascorbate peroxidase) activity increased in 1.4…1.5 times	(Kumar et al., 2013a)
Linum usitatissimum L.	*Agrobacterium tumefaciens* C58C1:pGV2260	*CHS* gene from *Petunia hybrida* X04080	Antioxidant activity and flavonoids content increased 5-fold	(Zuk et al., 2012)
Oryza sativa L.	*Agrobacterium tumefaciens* LBA4404	pMJ101 vector with *OsGS* gene	The content of glutathione increased 1.57 times, correlation of GSH/GSSG increased 5.2 times	(Park et al., 2017)
Hordeum vulgare L.	*Agrobacterium tumefaciens* AGL1	pCAMBIA13011 vector with *HvHGGT* gene	The content of δ-tocotrienol increased 2.44…2.66 times, the amount of β-tocotrienol increased 2.31…2.62 times; antioxidant activity increased by 17…18%; Trolox equivalent antioxidant capacity increased up to 33.46…33.84 μmol/g	(Chen et al., 2017)
Solanum tuberosum L.	*Agrobacterium tumefaciens* EHA 105	pCAM2300 vector with *GLOase* and *npt*II	The content of ascorbic acid increased by141%	(Hemavathi et al., 2010)
Arabidopsisth aliana (L.) Heynh.	*Agrobacterium tumefaciens* GV3101	pGEM-T vector with *EsWAX1* from *Eutrema salsugineum*	The content of ascorbic acid increased by 23…27%	(Zhu et al., 2014)

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Usually, the *A. rhizogenes*-mediated transformation is carried out in the following way:

a) The agrobacteria are cultivated overnight to reach the log phase of their growth.
b) Explants (e.g., leaves, internodes, or cotyledons) are prepared and scarred by sterile scalpels/blades.
c) The agrobacterial suspension is added to the explants. Sometimes this suspension is firstly centrifuged, the supernatant is discarded and the agrobacterial pellet is resuspended.
d) The cocultivation of explants and agrobacteria takes place using a standard medium (e.g. Murashige and Skoog medium) without any added antibiotics.
e) After a few days, the explants are transferred to the medium with antibiotics (e.g. cefotaxime) and are sub-cultured several times until the visible initiation of the hairy roots.
f) The transfer of individual hairy roots to obtain separate hairy root lines. If the used agrobacterial strain carries extra plasmid with a selective agent (such as resistance to antibiotics or herbicides), those lines are transferred onto the medium with antibiotics or selective agents.
g) The calculating frequency and efficacy of transformation. The frequency of transformation is the percentage of explants that were successfully transformed (we can see the initiation of hairy roots) out of all explants that were infected. The efficacy of transformation is the number that corresponds to the average quantity of hairy roots initiated per each successfully transformed explant.

After the elimination of agrobacteria from hairy root cultures, the PCR analysis is carried on usually. This helps to check the presence of *rol* genes (a marker of successful *A. rhizogenes*-mediated transformation), the presence of selective genes (if they are in the plasmid), and the absence of *vir* genes (a marker of the elimination of agrobacteria from hairy root cultures).

Regarding *A. tumefaciens*-mediated transformation, its protocol is generally similar to the one mentioned above. However, some changes need to be accomplished due to the nature of this bacterium, i.e. it does not initiate hairy root cultures that can grow on plant growth regulators-free medium, and *A. tumefaciens* strains nearly always carry selective agent genes. Thus, before the genetic transformation procedure, nutrient medium with plant growth regulators must be optimized individually for each studied plant. This medium should contain the correct ratio of auxins and cytokinins that induces direct regeneration of the studied plant. Otherwise, there will be impossible to obtain transformed plant lines, let alone calculate the frequency and efficacy of transformation.

As an example of successful *A. rhizogenes*-mediated transformation without any additional genes, you can look through Table 1. Table 2 gives examples of successful *A. tumefaciens*-mediated transformation with extra genes. Both tables list research papers focused on the initiation of new plant lines with boosted biosynthesis of secondary metabolites with antioxidant activities.

Other approaches for boosting secondary metabolites biosynthesis (and antioxidant activity) include the usage of *A. rhizogenes*-mediated transformation with binary vectors that carry additional genes. There are several engineering strategies aimed at increasing the availability of antioxidants that are synthesized de novo by plants. All of them are focused on the modification of endogenous plant metabolism and include such techniques (Zhu et al., 2013):

a) overexpression of a known rate-limiting enzyme that will mitigate a metabolic bottleneck, preferably using an enzyme without feedback inhibition;

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b) overexpression of multiple enzymes to ensure there is adequate flux throughout the entire pathway;
c) expression of regulatory proteins to coordinately induce an entire endogenous pathway without the introduction of heterologous enzymes;
d) suppression of a competitive pathway or branch point to ensure flux in the appropriate direction
e) creation/enlargement of a metabolic sink which reduces feedback inhibition and allows the desired product to accumulate stably.

The first two strategies are most commonly used. They support the introduction of various structural genes from other plants to create a recombinant biosynthesis pathway. Moreover, other parameters should be included. For example, for the overexpression of flavonoids, it is necessary to control structural gene overexpression or gene silencing, transcriptional regulation, flow control, and transporter overexpression (Wang et al., 2011).

Some examples of successful metabolic engineering in hairy root cultures are presented here. Park et al. (2021) showed that the maize transcription factor Lc (ZmLc) and *Arabidopsis* transcription factor PAP1 (AtPAP1) can enhance the production of flavonoid compounds baicalin, baicalein, and wogonin in hairy root cultures of *Scutellaria baicalensis*. The mechanism of enhancement is the comprehensive upregulation of flavonoid biosynthesis pathway genes (*SbPAL1*, *SbC4H*, *Sb4CL*, and *UBGAT* by ZmLc and *SbPAL1*, *SbPAL2*, *SbPAL3*, *SbC4H*, *Sb4CL*, *SbCHI*, and *UBGAT* by AtPAP1). Total flavonoid content increased by 322% using ZmLc and by 532% using AtPAP1.

Li et al. (2020a) focused on MYB transcription factors that play a key role in the phenylpropanoid biosynthetic pathway. Scientists constructed an overexpression system for four R2R3-MYBs in *Fagopyrum tataricum* resulting in tissue-specific expression of these genes and successive upregulation of phenylpropanoid biosynthesis.

Park et al. (2012) improved the production of rutin in *Fagopyrum esculentum* by overexpression of the flavonol-specific transcription factor AtMYB12 in hairy roots. This led to the increased expression of flavonoid biosynthetic genes (phenylalanine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate: CoA ligase, chalcone synthase, chalcone isomerase, flavone 3-hydroxylase, flavonoid 3'-hydroxylase, and flavonol synthase) and accumulation of rutin up to 0.9 mg/g dry weight.

Piao et al. (2021) investigated the expression of key regulators of anthocyanin biosynthesis in *Antirrhinum majus* hairyroots – genes *bHLH AmDelila* and *R2R3-MYB AmRosea1*. Ectopic expression of *AmRosea1* resulted in strongly enhanced anthocyanin accumulation by upregulation of the expression of the key target structural genes in their biosynthesis pathway. Another group (Qin et al., 2021) studied the anthocyanin regulatory gene *LcMYB1 Litchi chinensis*. The highly efficient transformation led to the induction of transgenic cultures with overexpression of *LcMYB1* and high content of anthocyanidins (3 mg/g FW) and proanthocyanidins (nearly 15 mg/g FW). Xu et al. (2020) studied the functions of anthocyanin-related regulatory gene *PpMYB10.1* and the strong activation of *PpUFGT* and *PpGST* downstream genes in hairy roots of peach. Li et al. (2016) focused on regulating anthocyanin and proanthocyanidin biosynthesis using bHLH complexes in *Medicago truncatula.* Complementation or overexpression of *MtTT8* resulted in the achievement of 100 μg/g FW of total soluble proanthocyanidins (e.g., epicatechin) and over 1600 μg/g FW of total insoluble proanthocyanidins in hairy roots. Yoshida et al. (2015) showed that MYB134 transcription factor overexpression leads to a high proanthocyanidin phenotype in poplar. They researched MYB182 transcription factor down-regulation of proanthocyanidin biosynthesis (shikimate pathway genes) by repressing both structural and regulatory flavonoid genes. Pang et al. (2013) studied proanthocyanidin pathway enzymes from tea (*Camellia sinensis*). Expression of the *CsLAR* (leucoanthocyanidin

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reductase) gene in tobacco led to the overproduction of anthocyanins and accumulation of higher levels of epicatechin, which may suggest the role of this gene in flavan-3-ol biosynthesis. Research by Terrier et al. (2009) concentrated on the study of proanthocyanidins in *Vitis vinifera*. Ectopic expression of VvMybPA transcription factors in hairyroots induced changes in the accumulation of proanthocyanidins up to 8 mg/g FW and activation of the flavonoid enzymes pathway, including anthocyanidin reductase and leucoanthocyanidin reductase 1, the specific terminal steps in the biosynthesis of epicatechin and catechin, respectively. An earlier study by Damiani et al. (1998) showed that adding maize gene *Sn*, which transactivates the anthocyanin pathway, initiated the pigmentation in 50% of transgenic lines.

Chen et al. (1999) transferred the farnesyl diphosphate synthase (*FDS*) gene into the hairy roots of *Artemisia annua,* which resulted in 3–4 times higher content of artemisinin than in the control hairy roots. Another study focused on artemisinin production boost was performed by Shi et al. (2017). They reported the simultaneous overexpression of four transferred artemisinin biosynthetic pathway genes in hairy roots: *ADS*, *CYP71AV1*, *CPR*, and *ALDH1*. Artemisinin contents increased significantly, with the highest being 3.4-fold higher than the control.

Bavage et al. (1999) studied the condensed tannins (proanthocyanidins) accumulation in *Lotus corniculatus* hairyroots. Dihydroflavonol reductase (*DFR*) gene from *Antirrhinum majus* was expressed, which resulted in several high-producing clones. The content of condensed tannins in hairy root lines exceeded control by 170%.

In addition, noteworthy advantages of using hairy roots are the possibility of extracellular secretion of expressed proteins (also known as rhizosecretion) and the ability to produce complex compounds at a large scale. This enables the expression of recombinant proteins by hairy root cultures grown in a bioreactor and their secretion into the culture medium under controlled and limited conditions. For example, mouse monoclonal antibodies from hairy tobacco roots were obtained for the first time in 1997 (Wongsamuth & Doran). Subsequently, other recombinant proteins were produced: green fluorescent protein (GFP) (Medina-Bolívar & Cramer, 2004), human acetylcholinesterase (Woods et al., 2008), mouse interleukin (Liu et al., 2009), sweetener thaumatin (Pham et al., 2012), human interferon-α2b (Luchakivskaia et al., 2012), human EPO (rhEPO) (Gurusamy et al., 2017), alpha-L-iduronidase (Cardon et al., 2019) and others. Thus, numerous heterologous proteins have been obtained using expression systems based on hairy roots, including antigens, antibodies, enzymes, and immunomodulators.

1.9. Effect of cultivation parameters on metabolites production in hairy roots

For the production of secondary metabolites (SMs) *in vitro*, researchers have to optimize all the needed parameters for the growth of hairy root cultures. Cultivation conditions and nutrient media are the main groups of the parameters (Fig. 8).

Certain cultivation conditions (temperature, lighting, agitation, aeration) have to be ensured to reach the optimum plant growth parameters. The usual constant temperatures for cultivation are around 24°C. Lighting is often provided as 16 hours of light per day. However, 24-hour lighting is also possible for some cultures in bioreactors. Agitation is necessary to ensure gas exchange (aeration) in liquid media.

Nutrient media are a source of all necessary substances for the growth and development of plant cultures. Standard media use provides easy reproducibility of experiments and the possibility to compare the results with the works of other researchers. Murashige & Skoog (MS) medium (1962) is the most commonly used standard nutrient medium for cultivating hairy roots and other plant cultures. Schenk & Hildebrandt (SH) medium (1972) and Gamborg (B5) medium (1968) are also used for this purpose.

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Any nutrient medium for *in vitro* plant cultures should have the following components: macrocomponents (N, S, P, Ca, K); microcomponents (Mn, Cu, B, Cl, Co, Mo, Zn, I); vitamins; source of Fe; source of Carbon. Not only the nutrient content of the medium is important but also its pH since plants grow only in a certain diapason of acidity. The pH from 5.5 to 5.9 is suitable for most species. Also, variable components are sometimes added: amino acids, growth regulators, and elicitors. Growth regulators are put in not only to maintain some cultures *in vitro* but also are added to obtain callus cultures (and suspension cultures from them), plant regeneration, rhizogenesis, or multiple shoot formation. Thus, by changing the composition of the nutrient medium, it is possible to obtain new plant cultures *in vitro* from already existing ones, as well as to investigate the totipotency of certain types of plants or to select and optimize media for their microclonal propagation.

The growth and production of SMs are often antagonistic due to competition for the same precursors. Sometimes it is necessary to change the cultivation parameters to achieve a high amount of the target compounds. Biosynthetic precursors and elicitors are widely used for this purpose. To familiarize ourselves with the concept of elicitors, we should revise the significance of secondary metabolites for plant cells and plant organisms in general. SMs participate in plant protection and communication with the environment. In addition, they are related to the color, taste, and smell of plants. Notably, they are also involved in plant responses to stress, both biotic (plant pathogens) and abiotic (temperature, drought, salinity, and UV light). Facing such stressors, plants can change their morphological characteristics (number of leaves or branches, leaf area, root height, and volume) along with their metabolism (Jan et al., 2021). Indeed, plants have a broad set of defense mechanisms that allow them to cope with stress conditions at the metabolomic level and enhance the accumulation of SMs during stress. Threat signals are recognized by plant receptors. This ensures a protective reaction, one of which is the accumulation of secondary metabolites. Transcription factors play an essential role in plant defense control by detecting stress signals and directing the expression of defense genes. Similarly, plant survival, longevity, and productivity depend on the increased synthesis of SMs known as elicitation. Various biotic (fungi, bacteria, etc.) and abiotic (exogenous hormones) elicitors are used to enhance the production of SMs in plants to protect them against stress stimuli. Thus, elicitors are compounds that, even in very small concentrations, contribute to the strengthening of secondary metabolism to protect the cell and the whole plant from various factors. Depending on the origin, elicitors can be classified into biotic and abiotic.

Biotic elicitors can be exogenous and endogenous. Exogenous elicitors come from pathogens (their enzymes, lysates, polysaccharides, and glycoproteins). Endogenous elicitors are produced by plants as a reaction to these pathogens (plant polysaccharides, proteins, and low-molecular compounds). Abiotic elicitors are subdivided into physical (e.g., UV light), chemical (inorganic compounds, e.g., sodium nitroprusside or cupric oxide), and hormonal (e.g., jasmonates and salicylic acid). Sometimes nanoparticles belong to the third group of elicitors and are called nanoelicitors. Salicylic and acetylsalicylic acid, chitosan, coronatine (a bacterial toxin), jasmonic acid and methyl jasmonate, pectin, and yeast extract are the main currently used elicitors. Elicitors could be added both *in vitro* and *ex vitro* individually and as a whole complex of elicitors simultaneously.

Now, let us review some specific examples regarding cultivation parameters and elicitors. Shinde et al. (2010) investigated the effects of media constituents on the production of the phytoestrogenic isoflavones daidzein and genistein in hairy roots of *Psoralea corylifolia*. It was found that supplementation of the medium with NH_4^+ and NO_3^- at a ratio of 2:1 increased the biomass and productivity of cultures. Increased levels of sucrose resulted in a higher yield of daidzein. However, decreased sucrose levels favored genistein production. The highest levels of daidzein (2.06% dry wt.) and genistein (0.37% dry wt.) were produced in the presence of low concentrations of PO_4^{3-}.

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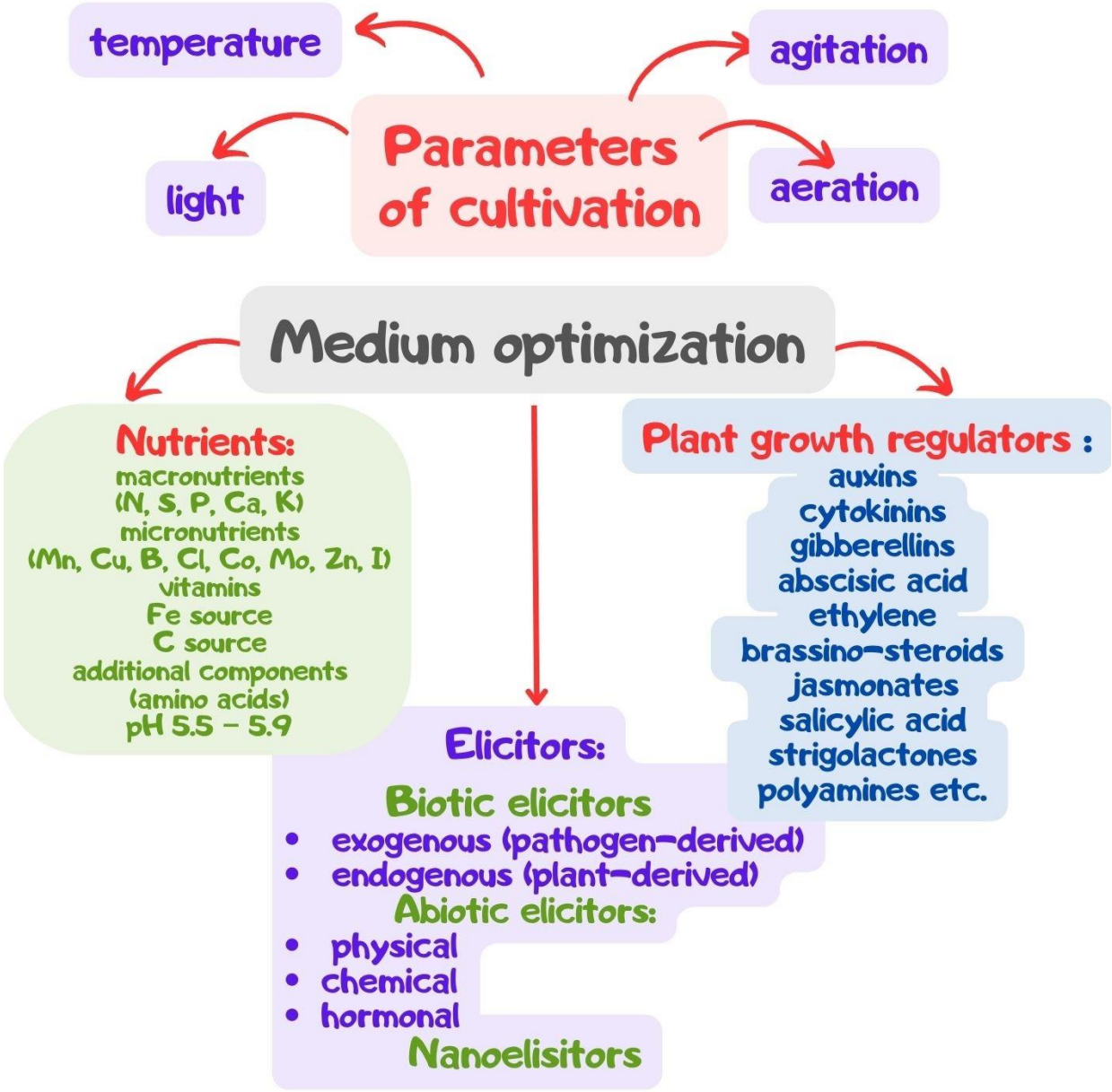

Figure 8 – Optimization strategies for secondary metabolites production in hairy root cultures *in vitro*

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Jia et al. (2017) showed that salt stress (85 mM NaCl) slightly induced isoflavone accumulation and salt tolerance in control (without additional genes) soybean hairy roots. However, there was a substantial reduction in the maximum root length, root fresh weight, and relative water content. Other cultures of hairy roots that carried the *hrGmIFS1* gene had a significantly higher content of isoflavones, and the mentioned above root growth parameters decreased much less. Then, this gene was transferred to tobacco plants. This resulted in greater plant height and leaf fresh weight after treatment with NaCl, thus confirming better salt tolerance. In addition, the leaf antioxidant capacity of tobacco became significantly higher.

Kim et al. (2012a) optimized nutrient medium for the best growth and flavone production in *Scutellaria baicalensis* hairy roots. Hairy root grown in full-strength SH medium was the highest (0.32 g/30 mL). The highest content of flavones baicalin, baicalein, and wogonin was detected in hairy roots grown in a half-strength B5 medium. Auxin treatments did not affect hairy root growth rates. However, flavone production was increased while using auxin indole acetic acid at 1 mg/L. Meanwhile, the highest levels of wogonin were observed in the presence of indolebutyric acid at 1 mg/L, followed by IAA at 0.1 mg/L.

Park et al. (2016) studied another parameter of medium optimization for flavonoid accumulation in hairy roots of *Scutellaria baicalensis* – the influence of different carbohydrates. Seven carbohydrate sources (sucrose, fructose, glucose, galactose, sorbitol, mannitol, and maltose) at a concentration of 100 mM were used for supplementation of a half-strength B5 liquid medium. Major flavones production was affected by sucrose, galactose, and fructose. Interestingly enough, different carbohydrates stimulated the production of different flavonoids. Sucrose was the optimal carbon source for the enhancement of baicalein production, fructose caused the greatest increase in baicalin accumulation, and galactose was the optimal carbon source for wogonin production.

Tusevski et al. (2013) showed that dark-grown and photoperiod-exposed hairy root cultures of *Hypericum perforatum* differed in phenolic acids, flavonols, flavan-3-ols, and xanthones accumulation. Light served as the elicitor for the quinic acid, kaempferol, and seven identified xanthones production. Moreover, two phenolic acids, three flavonol glycosides, and five xanthones were synthesized in light-exposed cultures *de novo*. On the other hand, dark-grown cultures had a higher content of flavan-3-ols (catechin, epicatechin, and proanthocyanidin dimers).

Zhang et al. (2018) investigated another type of light wave as a possible physical elicitor (far-red, white, blue, and red) for the enhancement of artemisinin accumulation in *Artemisia annua*. The results showed that red and blue light could enhance artemisinin production by inducing the expression of the genes involved in artemisinin biosynthesis (*ADS* and *CYP71AV1*).

Tashackori et al. (2016) examined the action of mycelium extract of *Piriformospora indica* as a biotic elicitor for hairy roots of *Linum album*. This extract enhanced the phenolic acids accumulation even after 12 h treatment and on lignans after 24 h treatment. The content of flavonols increased after 48 h treatment, and the levels of phenols and flavonoids were significantly enhanced after 72 h treatment (up to 422.69μg g^{-1} DW of phenols and 15.41μg g^{-1} DW of flavonoids). The activity of phenylalanine ammonia-lyase increased and peaked at 24 h after treatment. Another research by these authors (Tashackori et al., 2021) showed that digested cell wall of *P. indica* boosted this plant's metabolism and antioxidant activity. SOD (superoxide dismutase) and GPX (glutathione peroxidase) activity increased significantly. The expression of several genes (*PAL*, *CCR*, *CAD*, and *PLR*) was also enhanced.

Fattahi et al. (2021) explored the effect of methyl-β-cyclodextrins and coronatine (individually and combined) on tropane alkaloids accumulation in *Atropa acuminata* and *A. belladonna* hairy roots. In *A. belladonna* all the treatments reduced alkaloid production,

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while in *A. acuminata*, coronatine elicitation increased the scopolamine content 10-fold compared to the control (10.95 mg/g DW).

Jiao et al. (2017) investigated the action of immobilized food-grade fungi *Aspergillus niger* in a cocultivation system with *Astragalus membranaceus* hairy roots. The enhanced production of calycosin (730.88 ± 63.72 μg/g DW, 7.72-fold higher than in nontreated control) and formononetin (1119.42 ± 95.85 μg/g DW, 18.78-fold higher than in nontreated control) were achieved after 54 h cocultivation.

Vaccaro et al. (2017) showed that coronatine elicitation is effective for the accumulation of aethiopinone (abietane diterpenes) in *Salvia sclarea* hairy roots. Moreover, prolonged exposure to coronatine does not inhibit hairyroot growth, a common disadvantage of methyl jasmonate treatment. Coronatine addition yielded 24-fold higher content (up to 105.34 ± 2.30 mg/L) of aethiopinone after 28 days.

Jiao et al. (2018) studied ultraviolet radiation as a physical elicitor of flavonoid production in the hairy roots of *Isatis tinctoria*. Maximum flavonoid accumulation in the hairy roots treated with 108 kJ/m^2 dose of UV-B radiation increased 16.51-fold compared to that in the control. The antioxidant activity was enhanced as well. Moreover, the expression of the *chalcone synthase* gene was tremendously up-regulated (up to 405.84-fold), which may suggest the role of this gene in flavonoid accumulation.

Pitta-Alvarez et al. (2000) tested salicylic acid, yeast extract, $AgNO_3$, $CaCl_2$, and $CdCl_2$ on the tropane alkaloids accumulation in *Brugmansia candida* hairy roots. Salicylic acid enhanced alkaloids production from 2- to 12-fold, yeast extract increased alkaloids accumulation 3-fold (and scopolamine 7-fold), $AgNO_3$ – from 5- to 8-fold, $CdCl_2$ – from 3- to 24-fold (but was highly detrimental to growth) and $CaCl_2$ had no significant effect.

Naeem et al. (2020) investigated the action of salicylic acid and toxic arsenic (individually and combined) on the antioxidants and artemisinin accumulation in *Artemisia annua*. The addition of Arsenic at 45 mg kg^{-1} reduced the overall performance of plants, and at the same time enhanced the levels of antioxidants. Further addition of salicylic acid increased these antioxidants and the yield of artemisinin even more.

Peng et al. (2023) studied the effect of microwave and l-phenylalanine (individually and combined) on Tartary buckwheat sprouts. The best treatment condition was the combination of 250 W microwaves and 2.9 mmol L^{-1} l-phenylalanine. Specific activities of *PAL* (phenylalanine ammonia-lyase), *CHI* (chalcone isomerase), and *FLS* (flavonol synthase) in 5-day-old sprouts increased by 47.84%, 53.04%, and 28.02% compared with the control. The expression of the corresponding enzyme genes *FtPAL*, *FtCHI,* and *FtFlS1* increased by 39.84%, 24.78%, and 33.72% compared with the control.

Demirci et al. (2020) explored the effect of 24-epibrassinolide and l-phenylalanine on the root growth, total phenolics, total flavonoids, and caffeic acid derivatives accumulation in hairy roots of *Echinacea purpurea*. Treatment with 0.5 mg L^{-1} 24-epibrassinolide for 50 days resulted in the highest fresh root weight, dry root weight, and growth index, while l-phenylalanine had no significant influence on root growth. Moreover, 24-epibrassinolide at 1.0 mg L^{-1} concentration was found as the optimum for the accumulation of the highest total phenolics, total flavonoids, cichoric acid, caftaric acid, echinacoside, and *p*-coumaric acid contents.

Ghimire et al. (2019) examined the effect of methyl jasmonate and yeast extract elicitors on the enhancement of phenolic compounds accumulation in the hairy roots of *Aster scaber*. Higher fresh and dry root biomass was obtained after the elicitation with 100 mg/L of yeast extract. A more significant increase in total phenolics and flavonoid production was reached after the elicitation with methyl jasmonate. However, by the results of the research, both elicitors can be efficiently used for phenolic compounds accumulation: increased content of hydroxycinnamic acids, seven flavonols, seven

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hydroxybenzoic acids, vanillin, homogentisic acid, and resveratrol were found after addition of either elicitor.

Krzemińska et al. (2022) tested four elicitors (yeast extract, methyl jasmonate, trans-anethole, and cadmium chloride) on *Salvia bulleyana* hairy roots. They found that methyl jasmonate was the most effective. Phenolic compound accumulation increased by around 100% (up to 124.4 mg/g dry weight) after 72 h treatment. Strong antioxidant activity (scavenging of DPPH, ABTS, and superoxide anion radical) was also enhanced.

Gharari et al. (2020) explored the effect of methyl jasmonate, methyl-b-cyclodextrin, and chitosan (individually and combined) on the hairy roots of *Scutellaria bornmuelleri*. Accumulation of chrysin, wogonin, and baicalein increased 9.15…13.25 times after elicitation with methyl jasmonate combined with chitosan. Moreover, such a combination enhanced the expression of two significant genes involved in the flavonoid biosynthesis pathway, *FNSII-2*, and *MYB7*.

Pilaisangsuree et al. (2018) studied the action of cyclodextrin and methyl jasmonate (individually and combined) on the hairy roots of the peanut plant. Treatment with cyclodextrin enhanced the antioxidant activity to the highest Trolox equivalent antioxidant capacity value (28.30 ± 2.70 mM Trolox/g DW) and induction of CuZn-SOD (CuZn superoxide dismutase) and APX (ascorbate peroxidase) antioxidant enzymes activity. Co-treatment resulted in the highest phenolic content and, surprisingly, a decrease of CuZn-SOD, GPX, and APX activity.

Jain & Singh, S. (2015) examined the effect of pectin, yeast extract, and methyl jasmonate on the *Solanum melongena* hairyroots. Pectin treatment was found to be the most efficient to enhance solasodine production (151.23 μg/g DW) representing a 23-fold increase compared to control hairy roots and up to 88-fold compared to field-grown plants. Chung et al. (2018) investigated the influence of biologically synthesized silver nanoparticles on glucosinolates and phenolic compounds accumulation in the hairy roots of *Brassica rapa*. The study showed a significant increase in glucosinolates (glucoalyssin, glucobrassicanapin, sinigrin, progoitrin, gluconapin, 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin, glucobrassicin, neoglucobrassicin, and gluconasturtiin) and phenolic compounds (flavonols, hydroxybenzoic, and hydroxycinnamic acids) production in elicited hairy roots. Moreover, biological (antioxidant, antimicrobial, and anticancer) activities became significantly higher.

Nourozi et al. (2019) showed that elicitation of *Dracocephalum kotschyi* hairy root cultures with iron nanoparticles enhanced biomass accumulation. In addition, antioxidant enzyme activity and rosmarinic acid content increased (1194 μg g^{-1} FW) after 24 h of exposure to 75 mgL^{-1} iron nanoparticles. The content of xanthomicrol, cirsimaritin, and isokaempferide increased by 11.87, 3.85, and 2.27-fold, respectively.

All the mentioned above research studies show that it is possible to optimize nutrient medium and cultivation conditions plus find suitable elicitors for the maximum yield of target compounds in hairy root cultures. In addition, some elicitors using provides induction in the synthesis of diverse compounds *de novo*. Thus, hairy roots can be the unique and universal bioproducers of valuable phytochemicals.

1.10. CRISPR/CAS and hairy roots

Clustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats (CRISPR) are direct repeats and unique sequences in the DNA of many bacteria and most of known archaea that protect the cell from foreign genetic elements (bacteriophages, plasmids).

CRISPR systems and divided into two categories according to the configuration of their effector modules in the latest classification (Guo et al., 2022). Class 1 effectors utilize multi-protein complexes (type I, III, and rarely IV). Class 2 effectors rely on single-

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component effector proteins to disrupt target genes represented by Cas9, including types II, V, and VI. CRISPR/Cas9 belongs to type II and is currently the most widely used and thoroughly studied genome editing tool. It allows influencing precisely on biosynthetic pathways of the necessary target compounds.

Such high precision in gene editing is possible due to the induction of breaks (mostly double-strand breaks) at specific genome locations using site-directed nucleases (Bezie et al., 2021). Before CRISPR/Cas, scientists used zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The necessity to develop amino acid motifs that would bind to the desired target sequence with high affinity and specificity is the main disadvantage of these techniques (Scheben et al., 2017). It is very difficult, time-consuming, and expensive. Unlike ZFNs and TALENs, the CRISPR/Cas9 approach relies on homological base pairing between nucleic acids that ensure the targeting of the specific site in the genome. Thus, CRISPR/Cas is much more used nowadays due to its simplicity and high efficiency (Guo et al., 2022). The simplified scheme of producing CRISPR/Cas-gene edited medicinal plants is presented in Fig. 9. At first, the target gene should be chosen. Then, CRISPR/Cas vector is constructed. After that, the vector carrier is chosen and assembled. All methods of direct and *Agrobacterium*-mediated transformation could be used. Concerning agrobacterial transformation, both *A. tumefaciens* and *A. rhizogenes* can be applied. For that, a full genome sequence of the used agrobacterial strain (both chromosome and Ri-plasmid) is needed.

However, *A. tumefaciens*-mediated delivery of CRISPR reagents has its bottlenecks (Rodrigues et al., 2021): vector delivery to the plant cell nuclei and the subsequent plant regeneration. For both processes, the efficiency depends highly on species and genotype (Altpeter et al., 2016). To solve these two main problems, some recent progress was made with the use of morphogenic regulators that increase regeneration (Gordon-Kamm et al., 2019; Maher et al., 2020) and ternary plasmids for *A. tumefaciens* equipped with extra virulence genes (Anand et al., 2018; Sardesai et al., 2018; Bahramnejad et al., 2019; Desmet et al., 2020). Another possible solution is using *A. rhizogenes* and the induction of hairyroots. This method is promising, as hairy roots could be used without whole-plant regeneration, do not need additional plant growth regulators, and can quickly produce a lot of biomass. And if plant regeneration is still necessary, it is possible with the selected and optimized nutrient media. Moreover, there is a new approach in combination with transient *A. rhizogenes*-mediated *ex vitro* hairyroot induction and the CRISPR/Cas9 technique (Alok et al., 2017; reviewed in Niazian et al., 2022). It is a revolutionary method for fast and precise functional validation of root-related candidate genes.

Now, let us consider CRISPR/Cas-gene editing more precisely. Usually, the scientists choose target genes, editing which will cause the desired change in plant biosynthesis. For that, a full plant genome sequence and a thoroughly studied synthesis of the desired compound are needed. There are three pathways of secondary metabolites (SMs) biosynthesis: shikimate, acetate-mevalonate, and acetate-malonate (required mainly for primary metabolites synthesis – fatty acids and their derivatives, and only some aromatic SMs). All of them have their characteristics, and each of them is needed for the synthesis of secondary metabolites of a certain group. There are three main groups of SMs based on their structure and metabolic pathways: phenolic compounds, terpenoids, and nitrogen-containing compounds (alkaloids and glucosinolates). All their biosynthesis pathways are closely related (Borrelli et al., 2016; Mohaddab et al., 2022).

The shikimate pathway is linked with aromatic compound precursors synthesis in plants, fungi, and bacteria (but not animals). This biosynthetic pathway converts simple sugars from glycolysis and the pentose phosphate pathway into aromatic amino acids. Phenylpropanoids, precursors of such important SMs as flavonoids, coumarins, tannins, and lignins, are synthesized from these aromatic amino acids. In addition, the shikimate

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pathway provides the synthesis of nitrogen-containing SMs, such as alkaloids and glucosinolates, and some phytohormones, for example, β-indoleacetic acid.

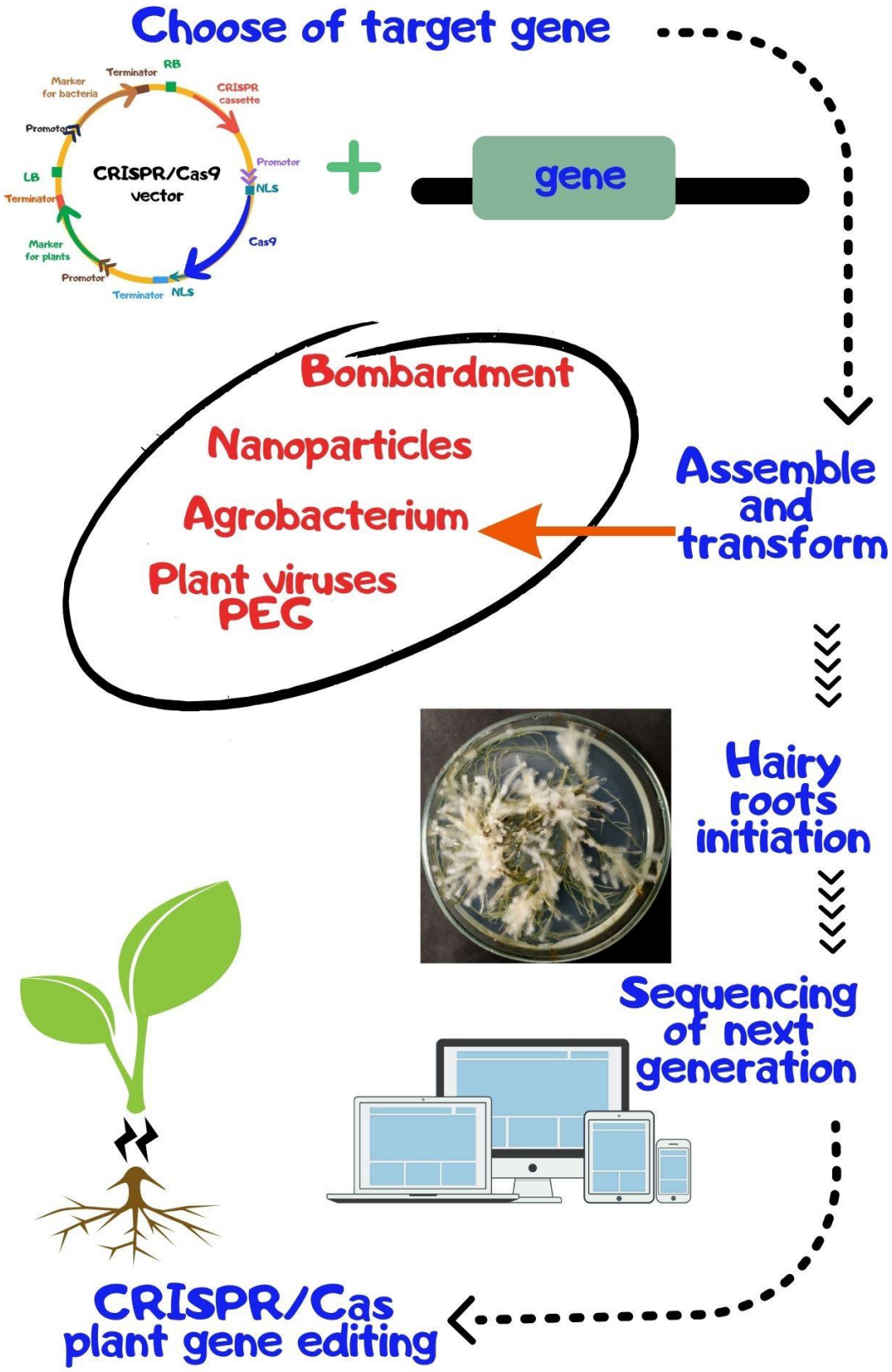

Figure 9 – A simplified scheme of generating CRISPR/Cas-gene editing medicinal plant lines (Guo et al., 2022)

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The acetate-mevalonate pathway is associated with isoprenoids – precursors of all terpenoids that are the components of plant essential oils. Terpenoids include: monoterpenes/terpenoids C10, sesquiterpenes C15, diterpenes C20, triterpenes C30, carotenoids/tetraterpenes C40 and polyterpenes (cytokinins, gutta-percha, gum) C>40. In addition to plants, the acetate-mevalonate pathway functions in many other organisms, from archaea to animals. Steroids, coenzyme Q10, cholesterol, sex hormones, and vitamins A and K are also synthesized in animals thanks to this biosynthetic pathway.

Therefore, using CRISPR/Cas-gene editing, it is possible to influence and modify these biochemical pathways, thereby increasing the value of a particular medicinal plant.

In addition, the process of designing CRISPR targets is greatly simplified by using web tools (Liu et al., 2019).

a) Resources for designing/cloning single CRISPR targets can be found at http://stuparcrispr.cfans.umn.edu/CRISPR
b) Resources for designing/cloning multiple CRISPR targets can be found at http://cfanspmorrell.oit.umn.edu/CRISPR_Multiplex.
c) Several other web tools are publicly available for target design. Vector information is also available at http://www.addgene.org.

When the target gene is determined, CRISPR/Cas9 vector has to be constructed and assembled (Fig. 10). Attention should be paid to the following four aspects (Kiryushkin et al., 2021):

a) which promoters to use to drive the expression of genes encoding different Cas nucleases;
b) which Cas-based system to use;
c) the design and construction of guide RNAs (gRNAs) and the assessment of their efficiency;
d) the choice of genes encoding markers for the identification of transgenic roots.

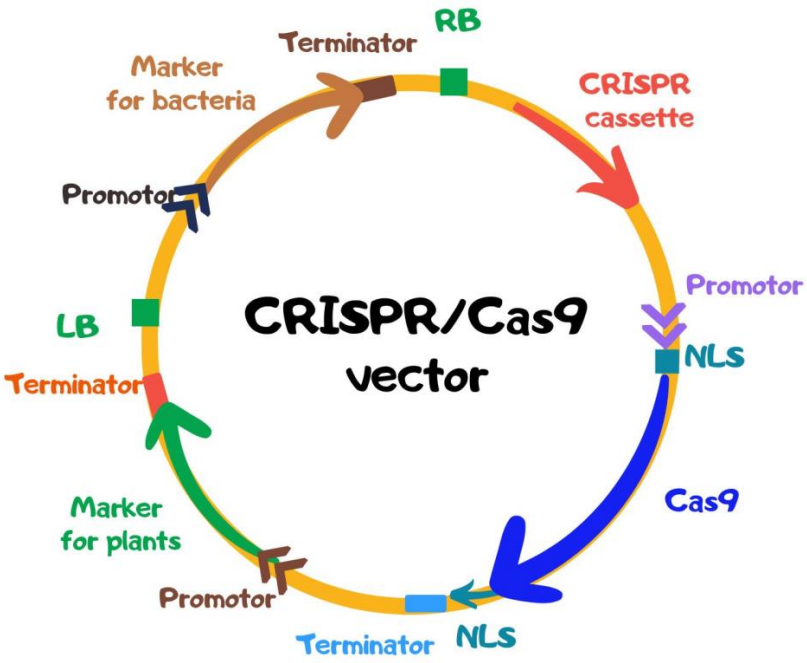

Figure 10 – Simplified map of a CRISPR/Cas9 vector. Vector components (clockwise): RB – right T-DNA border; CRISPR cassette – cassette expressing the guide RNA; NLS – nuclear localization signal; Cas9 – CRISPR-associated nuclease; LB – left T-DNA border (Kiryushkin et al., 2021).

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Cauliflower Mosaic Virus (p35S) promoters and their variants (2xp35S, 2xp35SΩ, p35SPPDK) are the most commonly used promoters. They are strong constitutive promoters used worldwide. Other promoters including Ubi (*ubiquitin* promoters from *Arabidopsis*, parsley, maize, rice, soybean, etc.) or pActin (promoter of the *Arabidopsis actin 2* gene), as well as inducible or organ- and tissue-specific promoters can also be used. Some enhancers may be added as well.

Cas nucleases, which can be utilized for genome editing, fall into the following three large groups:

a) those that can introduce double-stranded DNA breaks;
b) those that introduce single-stranded breaks;
c) those that do not introduce breaks.

The first group is the most common, and the Cas9 endonuclease of *Streptococcus pyogenes* and its modified versions are used for this purpose. Nickase form of Cas9 (nCas9) is used for single-stranded breaks introduction. The catalytically inactive (dead) form of Cas9 (dCas9) is used for editing without breaks.

The gRNA consists of the following two parts: 17–20-bp CRISPR RNA (crRNA) and 80-bp *trans*-activating crRNA (tracrRNA) (Kiryushkin et al., 2021). The crRNA is complementary to the target genomic DNA, and the conserved tracrRNA functions as a binding scaffold for a Cas nuclease (it is usually already incorporated in the CRISPR/Cas vector backbone).

Both selectable and screenable (visually) markers are commonly used for the reliable identification of transgenic hairy roots. Selectable markers include mostly genes that encode resistance to antibiotics (such as the *nptII* gene encoding neomycin phosphotransferase II that gives resistance against neomycin and kanamycin) or herbicides (such as *bar* gene encoding resistance to phosphinothricin/glufosinate). Widely used screenable markers include *β*-glucuronidase (GUS, a product of the *uid*A gene, which converts a soluble colorless substrate 5-bromo-4-chloro-3-indolyl-*β*-*D*-glucuronic acid into an insoluble colored product chloro-bromoindigo) and GFP. After the successful vector construction, plant transformation is carried on. Hairy root lines must be screened using PCR methods, and some metabolomic studies should be done. These analyses will help to verify whether the desired gene was inserted and what product was obtained.

Thus, CRISPR/Cas9 combined with hairy roots induction represents a fast and effective means of studying gene function (Bhattacharya et al., 2020; Kiryushkin et al., 2021; Jedličková et al., 2022; Niazian et al., 2022). It can be used for modification of plant traits when no stable transformation and regeneration procedures are available/possible or when the targeted characteristic is only observed in roots. Moreover, it results in much desirable quick obtaining of the transgenic biomass. As it was mentioned earlier, more than 20 strains of *A. rhizogenes* (wild-type or generated via genome engineering) (Bahramnejad et al., 2019) and more than 400 plant species (Porter, 1991) are currently available for hairyroot transformation. However, only 14 *A. rhizogenes* strains and about 30 plant species have been used in genome editing experiments to date (Kiryushkin et al., 2021). Some of these strains are transconjugant, i.e. they derive from other strains that were "cured" of pTi (usually strain C58). For example, the LBA1334 strain has the C58C9 chromosomal background and rifampicin resistance (Díaz et al., 1989).

The first paper on the CRISPR/Cas-mediated genome editing that could be used to investigate gene function in both plant protoplasts and whole plants was published in 2013 by Belhaj et al. Since then, scientists were interested in the genome editing capability concerning hairy roots, i.e. whether such a technique is applicable at all. These early successful studies include research on *Solanum lycopersicum* (tomato) (Ron et al., 2014 – first paper on CRISPR/Cas and hairy roots), *Glycine max* (soybean) (Michno et al., 2015; Cai et al., 2015), *Taraxacum kok-saghyz* (rubber dandelion) (Iaffaldano et al., 2016), *Arachis hypogaea* (peanut) (Yuan et al., 2019), and *Cichorium intybus* (chicory) (Bernard et al.,

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2019). Iaffaldano et al. (2016) and Bernard et al. (2019) showed that CRISPR/Cas9 could be used for the successful incorporation of studied genes. They also obtained the regeneration of whole genome edited dandelion and chicory plants. Nowadays, there are many more possible applications and research tasks solved by CRISPR/Cas9 genome editing in hairyroots (Fig. 11):

a) root nodule symbiosis investigation:
    - nodules formation (Shu et al., 2020; Tang et al., 2016; Fan et al., 2017; Fan et al., 2020; Lu et al., 2020; Xu et al., 2021; Wang et al., 2016; Wang et al., 2018; Yang et al., 2017);
    - Rhizobial early infection events (Zhu et al., 2020);
b) metabolic engineering:
    - tropane alkaloids metabolic pathway (Hasebe et al., 2021);
    - isoflavone metabolic pathway (Uchida et al., 2019);
    - glycyrrhizin (glycyrrhizic acid) content (Zhang et al., 2021; Wang et al., 2021);
    - camptothecin production (Shi et al., 2020);
    - salicinoid content (Fellenberg et al., 2020);
    - gallic acid and its glicosides content (Changetal., 2019);
    - tashinones content (Li et al., 2017);
    - lithospermic acid and its precursors (Zhou et al., 2018);
    - phenolic acid and tashinones content (Deng et al., 2020);
    - salvinalonic acid B, tashinones, rosmarinic and caffeic acids content (Hao et al., 2020; Yu et al., 2022; Zhou et al., 2021);
    - lignin biosynthesis pathway (Zhou et al., 2021);
    - α-tomatine and dehydrotomatine biosynthesis pathway (Akiyama et al., 2019; Swinnen et al., 2020);
    - α-solanine and α-chaconine biosynthesis pathway (Nakayasu et al., 2018);
    - honospermydine content (Zakaria et al., 2021);
c) root development:
    - root hairs formation (Kirchner et al., 2017);
    - lignin biosynthesis (Dai et al., 2020);
    - root to shoot Na^+ and K^+ transport (Niu et al., 2020);
    - endodermis development (Triozzi et al., 2021);
    - meristem size (Ron et al., 2014);
d) investigation of resistance to abiotic stress conditions:
    - sensitivity to the drought (Du et al., 2020);
    - sensitivity to salinity (Li et al., 2021; Sun et al., 2021);
e) investigation of resistance to the biotic stress conditions:
    - resistance to soybean cyst nematode (Butler et al., 2021; Dong et al., 2022);
    - resistance to *Phytophthora sojae* (Yu et al., 2022);
    - interaction between hemiparasitic plant *Phtheirospermum japonicum* and *Arabidopsis* (Greifenhagen et al., 2021);
    - tolerance to *Candidatus Liberibacter solanacearum* (Irigoyen et al., 2020);
f) supervirulent *A. rhizogenes* strains obtaining:
    - comparative analysis of agrobacterial genomes and identification of the parts that affect their virulence (Anand et al., 2018; Sardesai et al., 2018; Bahramnejad et al., 2019; Desmet et al., 2020);
g) commercial traits development:
    - plant architecture, photoperiod and circadian rhythmicity, storage proteins, seed oil, been flavor-free soybeans, seed weight, and organ size (reviewed in Xu et al., 2020 and Xu et al., 2022);
h) improvement of flowering ornamental plants (Pérez de la Torre et al., 2018).

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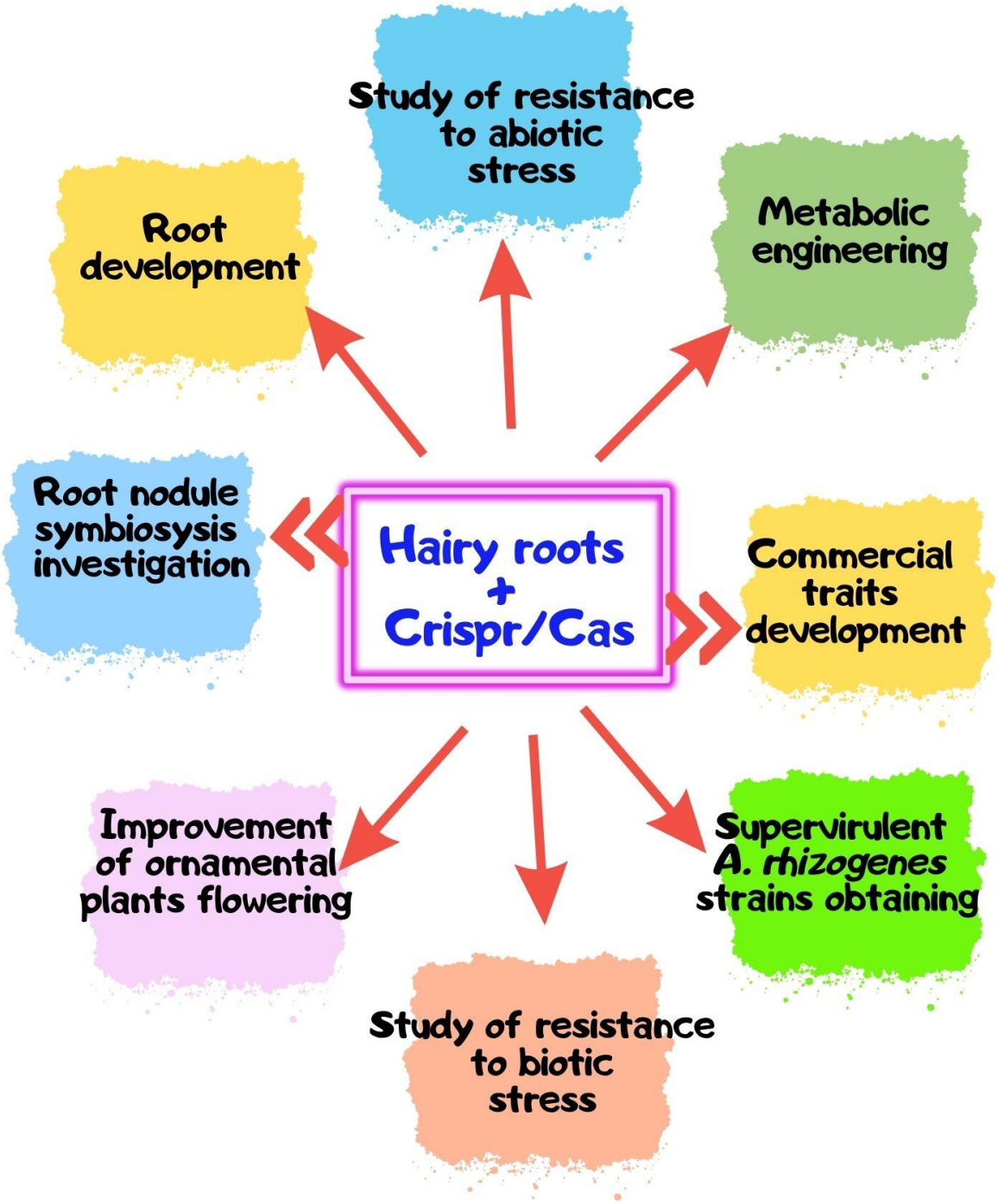

Figure 11 – Possible applications and research tasks solved using CRISPR/Cas9 genome editing in hairy roots

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1.11. Hairy roots of medicinal plants

Medicinal plants, as seen from their common name, are used directly to treat diseases or obtain certain biologically active compounds with a therapeutic effect. It should be noted that not only so-called medicinal plants synthesize biologically active compounds. Almost every plant is medicinal because it contains chemical components with different bioactivity. These components can be secondary metabolites, reserve, or protective chemicals. However, the amount of such valuable compounds in plants is usually low. It is possible to increase their concentration by stimulating synthesis in plant cells in various ways since even growing conditions significantly affect biosynthesis processes.

At the same time, hairy root construction can serve as an interesting and promising approach to obtaining producers of valuable medicinal compounds from plant raw materials. Biotechnological usage of hairy roots is possible due to their rapid growth feature. They can be grown in bioreactors avoiding the vagaries of the weather. In addition, scientists can choose among several lines of hairy roots the one with the highest production of bioactive components. That is why, for more than forty years, researchers have been adapting methods of genetic transformation to obtain hairy roots of various species, developing technologies for growing them in bioreactors, investigating the peculiarities of the biosynthesis of chemical compounds in these roots and methods of their extraction.

The list of plant species that are currently used to obtain hairy roots is very large. Some of them are presented in Table 3. Among them are well-known and rare plants.

Table 3 – Plants used for obtaining hairy root cultures

Species of plants	**Compounds**	**References**
Ambrosia artemisiifolia L.	thiarubrine A	Bhagwath et al., 2000
Anisodus acutangulus C.Y.Wu & C.Chen	tropane alkaloids	Kai et al., 2011
Arachis hypogaea L.	resveratrol, arachidin	Abbott et al., 2010
Artemisia annua L.	artemisinin	Putalun et al., 2007
Artemisia vulgaris L.	flavonoids, essential oil	Matvieieva et al., 2019a; Sujatha et al., 2013
Artemisia tilesii Ledeb.	flavonoids	Matvieieva et al., 2020
Astragalus membranaceus L.	triterpenes, astragalosides and flavonoids	Park et al., 2015; Jiao et al., 2015
Atropa belladonna L.	alkaloids	Bonhomme et al., 2000; Bensaddek et al., 2001 Bonhomme et al., 2000
Brassica rapa ssp. Rapa L.	glucosinolates	Chung et al., 2016a
Brugmansia candida Pers.	cadaverine	Pitta-Alvarez et al., 2000; Carrizo et al., 2001
Cajanus cajan (L.) HUTH	isowighteone	Gajurel et al., 2022
Calendula officinalis L.	Saponins (oleanane-type)/ cholesterol, campesterol, isofucosterol, sitosterol, stigmasterol	Alsoufi et al., 2019a; Alsoufi et al., 2021; Alsoufi et al., 2019b
Catharanthus roseus (L.) G.DON,	flavonoids, monoterpenoid indole alkaloids	Traverse et al., 2022; Chung et al., 2009; Van Moerkercke et al., 2015
Species of plants	Compounds	References

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Species of plants	Compounds	References
Centella asiatica (L.) URB	centellasaponins, asiaticosides, madecassosides/ campesterol, cholesterol, sitosterol, stigmasterol	Kim et al., 2010
Cichorium intybus L.	inulin	Bais et al., 2001; Tabatabaee Bais et al., 2021
Cucumis anguria L.	phenolic compounds	Yoon et al., 2015
Datura candida Safford	alkaloids	Christen et al., 1991.
Dracocephalum kotschyi Boiss	rosmarinic acid	Fattahi et al., 2013
Echinacea purpurea (L.) MOENCH	chicoric acid	Salmanzadeh et al., 2019
Erigeron breviscapus (Vaniot) Hand.-Mazz	scutellarin	Chen et al., 2018
Ferula pseudalliacea Rech.	farnesiferol B	Khazaeiet al., 2019
Isatis tinctoria L.	flavonoids	Gai et al., 2015
Isatis indigotica L.	lariciresinol	Ma et al., 2017
Gentiana dinarica Mann. Lerch.	xanthone	Krstić-Milošević et al., 2017
Glycine max L.	flavonoids	Jiang et al., 2010
Glycyrrhiza uralensis Fisch	flavonoids	Zhang et al., 2009; Yin et al., 2020
Gossypium hirsutum L.	gossypol	Vermaet al., 2009
Helicteres isora L.	diosgenin	Kumar et al., 2014
Hyoscyamus albus L.		Christen et al., 1992.
Hyoscyamus niger L.	tropane alkaloids	Zhang et al., 2007
Hyoscyamus reticulates L.	tropane alkaloids	Zeynali et al., 2016
Lawsonia inermis L.	tannin	Bakkali et al., 1997.
Ligularia fischeri Turcz. f. spiciformis (Nakai)	polyphenolic compounds	Ansari et al., 2019
Lopezia racemosa Cav.	organic extract	Vargas-Morales et al., 2022
Macleaya cordata (WILLD.) R.BR.	alkaloids	Huang et al., 2018
Momordica charantia L.	Phenolic compounds	Chung et al., 2016b
Momordica dioica Roxb. ex. Willd	phenolic compounds	Thiruvengadam et al., 2016
Nicotiana tabacum L.	alkaloid nicotine	Zhao et al., 2013
Ononis spinosa L. and *Ononis arvensis* L.	medicarpin glucoside and sativanone glucoside	Gampe et al., 2021
Ophiorrhiza pumila Champ. ex Benth.	Camptothecin	Shi et al., 2020
Panax ginseng C.A.Mey.	ginsenoside	Inomata et al., 1993; Overfield et al., 2003; Kochan et al., 2018
Pelargonium sidoides Cand.	coumarin	Yousefian et al., 2020
Platycodon grandifolium (Jacq.) A. DC.	platycodins (oleanane-type)/ α-spinasterol	Kim et al., 2013

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Species of plants	Compounds	References
Polygonum multiflorum (Thunb.) Moldenke	phenolic compounds	Ho et al., 2018
Polyscias filicifolia Bailey	chlorogenic acid caffeic and ferulic acid derivatives	Śliwińska et al., 2021
Portulaca oleracea L.	noradrenalin	Pirian et al., 2012
Psoralea corylifolia L.	flavonoids	Bourgaud et al., 1999; Shinde et al., 2009
Pueraria phaseoloides (Roxb.) Benth.	puerarin, deoxymiroestrol	Shi et al., 2003; Udomsin et al., 2019
Punica granatum L.	tannin	Ono et al., 2012
Rauwolfia serpentine (L.) BENTH. ex KURZ,	alkaloids	Mehrotra et al., 2015
Rhodiola crenulata Ohba	salidroside	Lan et al., 2013
Rhodiola rosea L.	salidroside, rosavinoids rosavin, rosarin, and rosin	Lütken et al., 2017
Rubia yunnanensis Diels	cyclopeptides and quinones	Miao et al., 2021
Salvia miltiorrhiza Bunge	tanshinone; rosmarinic acid	Wu et al., 2008; Liang et al., 2012; Xiao et al., 2011
Saussurea medusa Maxim	jaceosidin	Zhao et al., 2004
Scutellaria baicalensis Georgi	flavonoids	Elkin et al., 2018; Stojakowska et al., 2000; Park et al., 2011; Park et al., 2021
Scutellaria lateriflora L.	baicalin, baicalein, wogonin	Tuan et al., 2018
Scutellaria bornmuelleri Hausskn. ex Bornm.	flavonoids	Gharari et al., 2020
Silene vulgaris (Moench) Garcke	sapogeninsin	Kim et al., 2015
Silybum marianum L.	flavonolignan, silymarin	Alikaridis et al., 2000; Rahnama et al., 2008; Khalili et al., 2010
Solanum trilobatum L.	solasodine	Shilpha et al., 2015
Stephania suberosa Forman	dicentrine	Putalun et al., 2009
Tanacetum parthenium (L.) SCH.BIP.	parthenolide	Pourianezhad et al., 2019
Taxus cuspidate SIEBOLD et ZUCC. ex ENDL.	paclitaxel	Kim et al., 2009
Trachyspermum ammi L.	thymol	Vamenani et al., 2020
Tribulus terrestris L.	ß-carboline alkaloids	Sharifi et al., 2014
Trifolium pratense L.	isoflavones	Kumar et al., 2018
Trigonella foenum-graecum L.	diosgenin	Zolfaghari et al., 2020
Valeriana officinalis L.	sesquiterpenoids	Ricigliano et al., 2016
Vitis rotundifolia Michx.	stilbenoids	Ñopo-Olazabal et al., 2013
Vitis vinifera L.	resveratrol, stilbene	Hosseini et al., 2017; Tisserant et al., 2016
Withania coagulans (Stocks) Dunal	withanolide	Mirjalili et al., 2009; Murthy et al., 2008

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2. MATERIALS AND METHODS

Plants in vitro cultivation

To introduce plants *in vitro*, the seeds were sterilized in a 25% solution of the commercial preparation "Bilyzna" (Ukraine) for 10 minutes, washed three times for 5 minutes with sterile distilled water, and cultivated on the surface of a 1/2MS solidified medium (Murashige and Skoog medium, Duchefa, with half strength of macroelements) at a temperature of 24°C and 16-hour illumination.

Plant genetic transformation

Leaves of 10-14-day-old seedlings or *in vitro* cultivated plants were used as explants. *A. rhizogenes* strains were grown at the standard conditions on an LB liquid culture medium containing 10 g/l casein hydrolyzed, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0 overnight at 28°C in the dark on a shaker (220 rpm). For transformation, explants were cocultivated with bacterial suspension for 30 min (density 2.00-2.50 at 600 nm) and then were transferred to the selective media to induce hairyroot formation. MS medium with half strength of macroelements and 600mg/l cefotaxime was used for hairyroots formation in the case of *A. rhizogenes* – mediated transformation.

Hairy roots subcultivation

The roots from the collection were subcultivated on 1/2 MS solidified medium (half strength Murashige and Skoog medium) at 24°C and 16-hour illumination.

Plant material for chemical study

Hairy root lines from the collection of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used as the plant material for the study. The roots were grown at +24 °C for 2-4 weeks on the solidified nutrient 1/2 MS medium with the addition of sucrose at a concentration of 20 g/l.

PCR analysis

Genome DNA was extracted from the green leaves of sterile plants or hairy roots according to the CTAB method. PCR analysis of genome DNA was performed on Mastercycler personal 5332 amplifier (Eppendorf) using primers for target and selective genes. The amplification conditions were as follows: primary denaturation at 90°C, 3 min; 30 cycles of amplification (94°C, 30 sec; 56-67°C, 30-40 sec; 72°C, 30 sec); final polymerization at 72°C, 5 min.

Weight gain

The weight gain was determined after 2-4 weeks of cultivation at a temperature of +24°C on Murashige and Skoog medium with a sucrose concentration of 20 g/l. The roots were separated from the medium, washed with deionized water, dried with filter paper, and weighed on Sartorius scales with a standard deviation of ± 0.005 g.

Total flavonoids content assay

Determination of the content of flavonoids was carried out according to the method with $AlCl_3$. To prepare the extracts, the roots were separated from the medium, washed with deionized water, dried using filter paper, weighed, and homogenized in 70% ethanol. The homogenate was centrifuged in an Eppendorf Centrifuge 5415 C at 15 000 g for 10 min. The reaction mixture contained 0.25 ml of extract supernatant, 1 ml of deionized water, 0.075 ml of 5% $NaNO_2$ solution. After standing for 5 minutes, 0.075 ml of 10% $AlCl_3$ solution was added and held for another 5 minutes. 0.5 ml of 1M NaOH and 0.6 ml of deionized water were added. Absorption was determined at λ = 510 nm on a Fluorate-02-Panorama spectrofluorimeter. The calculation of the total content of flavonoids was carried out in the rutin equivalent (RE).

Antioxidant activity assay

The antioxidant activity of ethanol extracts of hairy roots was studied using the DPPH test. The optical density of the solutions was measured at a wavelength of λ = 515

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nm on a spectrofluorimeter. The radical scavenging activity (RSA, %) was calculated according to the following formula:

$$RSA = 100\ (OD_0 - OD_1)\ /\ OD_0,$$

where OD_0 – optical density of the DPPH solution, OD_1 – optical density of the of the reaction mixture after carrying out the reaction with DPPH. The effective concentration (EC_{50}) was calculated as the fresh weight of the root (mg FW) required to scavenge 50% of DPPH in the reaction with the radical (RSA = 50%).

Reducing power assay

Reducing power was studied according to the ability of root extracts to reduce iron ions Fe^{3+} to Fe^{2+}. The reaction mixture contained: 0.312 ml of 0.2 M phosphate buffer (pH 6.6); 0.312 ml of 1% potassium hexacyanoferrate(III); and ethanol root extract, the concentration of which was successively reduced. The cuvettes were incubated in a water bath at 50 °C for 30 min. After that, 0.312 ml of 10% trichloroacetic acid, 1.25 ml of deionized water, and 0.25 ml of 0.1% iron(III) chloride were added to the reaction mixture. The optical density was measured at a wavelength of λ = 700 nm on a spectrofluorimeter. Reducing power was characterized by the effective concentration parameter ($EC_{0.5}$ corresponding to root weight (mg FW) required to obtain OD = 0.5.

SOD activity determination

The activity of the superoxide dismutase was studied using nitro blue tetrazolium chloride. Root material (100 mg) was placed in an cuvettes and triturated with 1 ml 50-mM Tris-HCl buffer (pH=8.0), then centrifuged at 13000 g (4°C) for 15 minutes. The reaction was carried out in Eppendorf tubes (1.5 ml) with 10 µl of supernatant, 540 µl of 50-µM Tris-HCl buffer, 130 µl of 65 mM methionine, 47 µl of 630 µM of nitro blue tetrazolium chloride, 12.5 µl of 1 mM of riboflavin. One tube for each specimen was left in the dark another was held under the influence of a white lamp light (fluorescent lamp T5 / G5 model ELI - 230A - T5-8W) for 5 minutes in a thermostat at 26° C. Adsorption of the combined reaction mixture against the unleaded reaction mixture was measured at 550 nm using BioPhotometer (Eppendorf) v.1.35. The zero-sample contained all of these components except plant extract.

UPLC-ESI-UHR-Qq-TOF-MS analysis

For the analysis of multiple compositions in the ethanol extract of roots, the UPLC system (Dionex Ultimate 3000) was coupled with an Ultra-High Resolution Qq-Time-of-Flight (UHR-QqTOF) mass spectrometry equipped with an electrospray ionization (ESI) interface (Bruker Impact II). The mass spectrometer was operated in the negative ESI mode with a Duo-Spray source, and the mass scan range was set at m/z 50–2500 for the TOF-MS scan using a resolution of 2700. The following parameter conditions were used: ion spray voltage, 3500 V; ion source heater; curtain gas, 25 psi; collision energy, 10 eV; declustering potential 100. The analyst TF software (version 1.7) combined with the information dependent acquisition package was used to acquire the MS data. The mobile phase was composed of 0.1% formic acid in water (elution A) and methanol (elution B) using a gradient elution of 30% elution B (0–5 minutes), from 30% to 50% of elution B (5 and 20 minutes), from 50% to 90% elution B (20–40 minutes), and from 90% to 100% of elution B (40–45 minutes).

Artemisinin content study

Artemisinin and its co-products (ART) content were studied by HPLC–MS system. Chromatographic analysis was performed using High–efficiency liquid chromatograph Agilent 1200, Agilent Technologies, USA. The separation was carried out by isocratic method using ZORBAX SB–C18 2.1 mm × 150 mm, 3.5 µm (Agilent Technologies, USA) column with a 30°C. Water with methanol and acetonitrile (30/20/50 v/v) was used as a mobile phase with 0.4 ml min–1 flow–rate. The samples were analyzed separately

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according to their retention times. Their spectrum was compared with the spectrum of the artemisinin standard (Sigma–Aldrich, catalog number 63968–64–9).

Preparation of nanoparticle colloid solutions

The hairyroot ethanol extracts were used to obtain colloid solutions of silver nanoparticles (AgNPs). Namely, 0.3 ml of the extracts and 3 ml of 1 mM $AgNO_3$ were mixed thoroughly. After that, all the solutions were incubated for one hour in a water bath at +80°C to reduce Ag^+ to Ag^0.

Characterization of AgNPs

Transmission electron microscopy (TEM) was used to examine the size and morphology of the synthesized NPs. The image was done on a TEM1230 JEOL (Tokyo, Japan) with an acceleration voltage of 80 kV. The samples for TEM investigations were prepared by drying 0.03…0.05 ml of colloidal solution dropwise on Cu-grids with a previously carbon-coated film at room temperature.

AgNPs spectrophotometry assay.

The absorbance of the samples (colloid solutions of AgNPs) was measured automatically in the wavelength range of 300…600 nm (Fluorat-02-Panorama spectrofluorimeter) right after the incubation in a water bath, then in five and nine days to observe the dynamics of spectra changing. UV-vis spectra of the samples were obtained using the PanoramaPro software.

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3. THE RESULTS OF PLANT GENETIC TRANSFORMATION

3.1. Bioactivity of the hairy roots from the collection of the Institute of Cell Biology and Genetic Engineering NAS of Ukraine

The collection of hairy roots of medicinal plants presented in the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine includes almost one hundred specimens of sixteen species (Fig. 12). Some of these hairy roots are subcultured for nearly 20 years under sterile conditions in a thermostatically controlled room with a temperature of 24˚C. The presence of roots obtained due to wild strains of agrobacteria and other roots obtained after the transformation using bacteria that had additional plasmids with different target genes is a specific feature of the collection.

Such diversity makes it possible to study the physiological and biochemical characteristics of the collection specimens. Collection samples can also be used to study the pleiotropic effects of genetic transformation and the influence of various transferred genes on plant cells and organs. These studies have both a purely fundamental orientation and practical interest, as they allow us to select among a large of potentially valuable samples from a technological point of view, which can find a practical application for the production of compounds valuable for medicine. The collection specimens were used in the work financed by Ukrainian and international grants.

The collection of hairy roots was used to study the effect of genetic transformation on different aspects of the functioning of plant cells. To conduct this study, we evaluated the changes in hairy roots compared to the control (mother) plants cultivated in the same *in vitro* conditions. Such parameters were compared: total content of flavonoids; antioxidant activity; reducing activity; antimicrobial activity; activity of plant ferments (catalase and superoxide dismutase). The results of the study indicated the differences between the studied hairy root lines obtained using the same plants and the same bacteria. This effect was probably due to the introduction of bacterial genes (*rol*) in different sites in hairyroot clones, which were the independent transformation events. In many cases, the stimulating effect of the genetic transformation was observed.

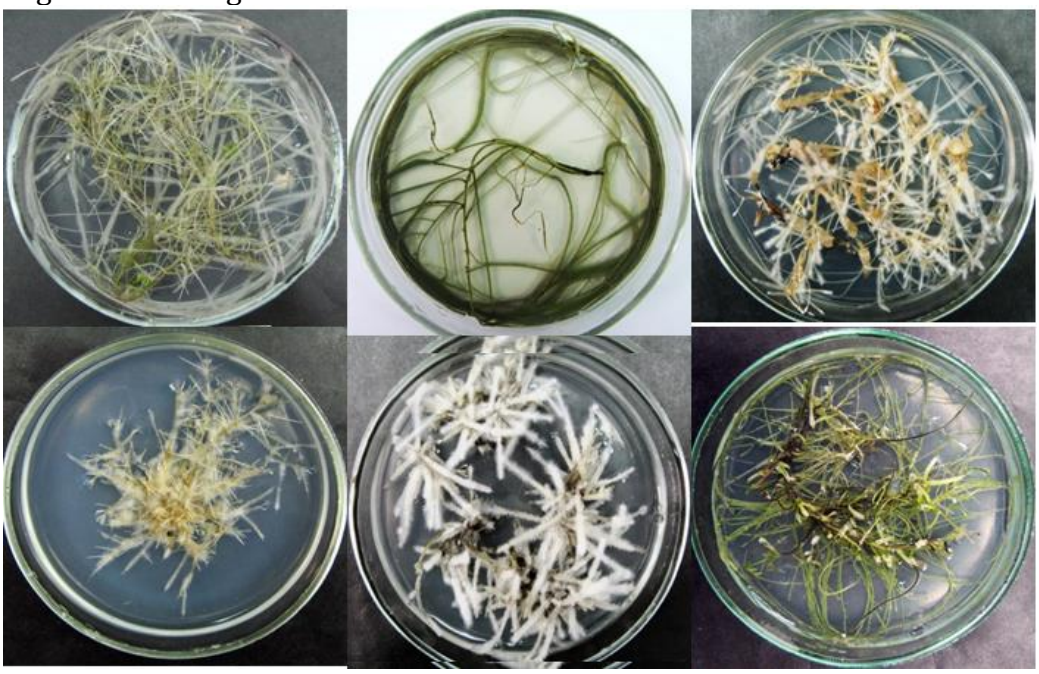

Figure 12 – The samples from the collection of hairy roots of medicinal plants (Laboratory of Adaptational Biotechnology, Institute of Cell Biology and Genetic Engineering NAS of Ukraine, Kyiv, Ukraine)

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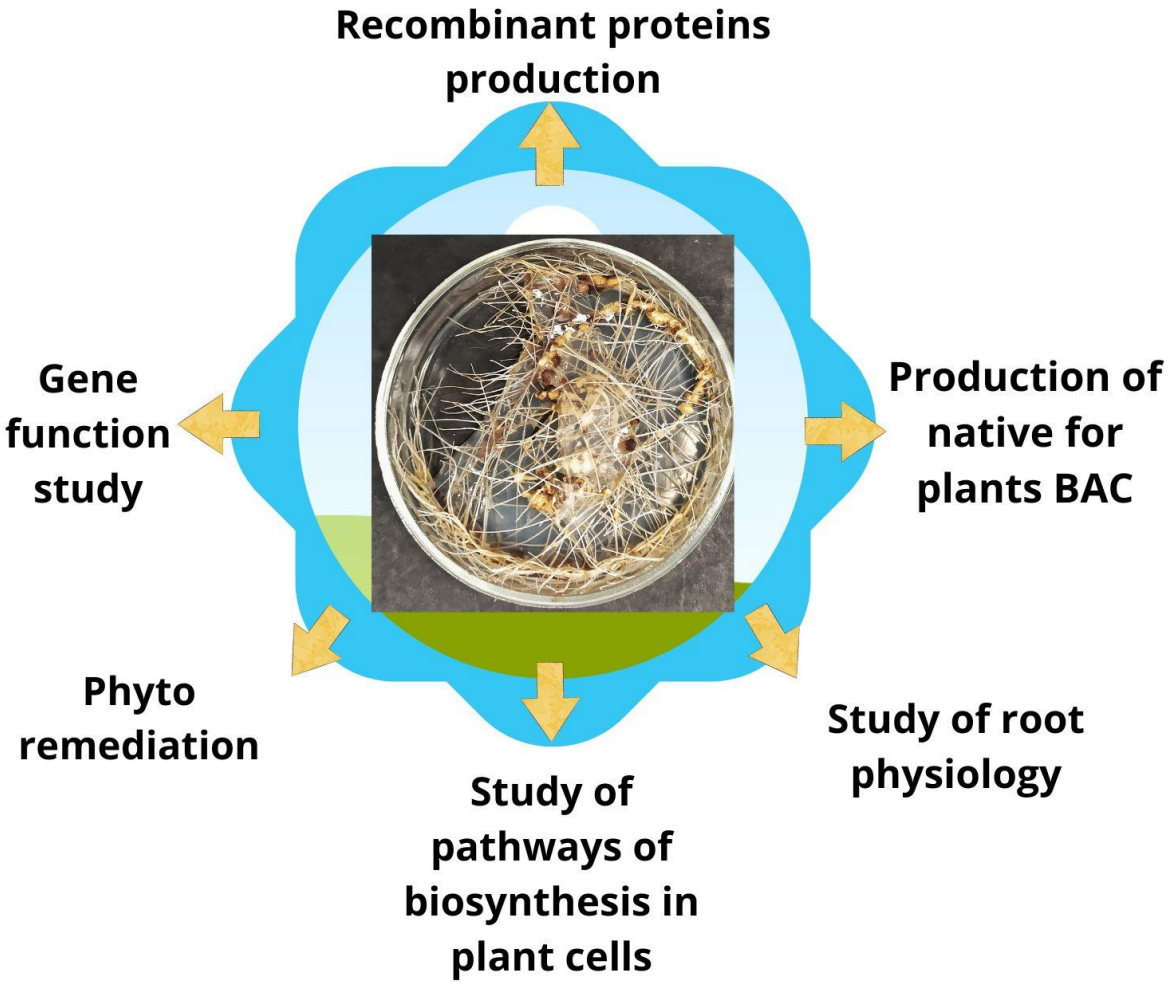
Application of "hairy" roots



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3.2. Fructans content

The genetic transformation using *Agrobacterium rhizogenes* affected fructans synthesis in hairy roots. hairyroots of different plant species accumulated fructans at 21-190 mg/g dry weight (Fig. 13). Comparative assessment of fructan content in the transgenic roots and control plants of *Artemisia* spp – *A. annua, A. tilesii, A. dracunculus, A. ludoviciana,* and *A. absinthium* was carried out (Duplij et al., 2017). The highest fructan content was found in the control leaves and roots of *A. annua* (39.4 and 32.5 mg/g fresh weight, respectively), the lowest – in the hairy roots of *A. dracunculus*, obtained by Agrobacterium rhisogenes-mediated transformation using A4 wild strain (6.4 mg/g) and in the leaves of the control *A. ludoviciana* plants (6.5 mg/g). The widest variation of fructan content was measured in samples of *A. annua*, the lowest – in *A. dracunculus* (Fig. 14).

The effect of some growth regulators Ivin (N-oxide 2,6-dimethyl pyridine), Emistim, Biolan, and Charkor ("AgroBioTech", Ukraine) on *Cichorium intybus* L. hairy roots growth and fructans accumulation was studied (Tsyganкova et al., 2013).

Addition of regulators to the MS medium at a concentration of 2.5-10.0 µl/l stimulated root growth. Fructans content in hairy roots increased 7.0-35 fold and depended on the type of regulator and hairyroot line used in the experiment.

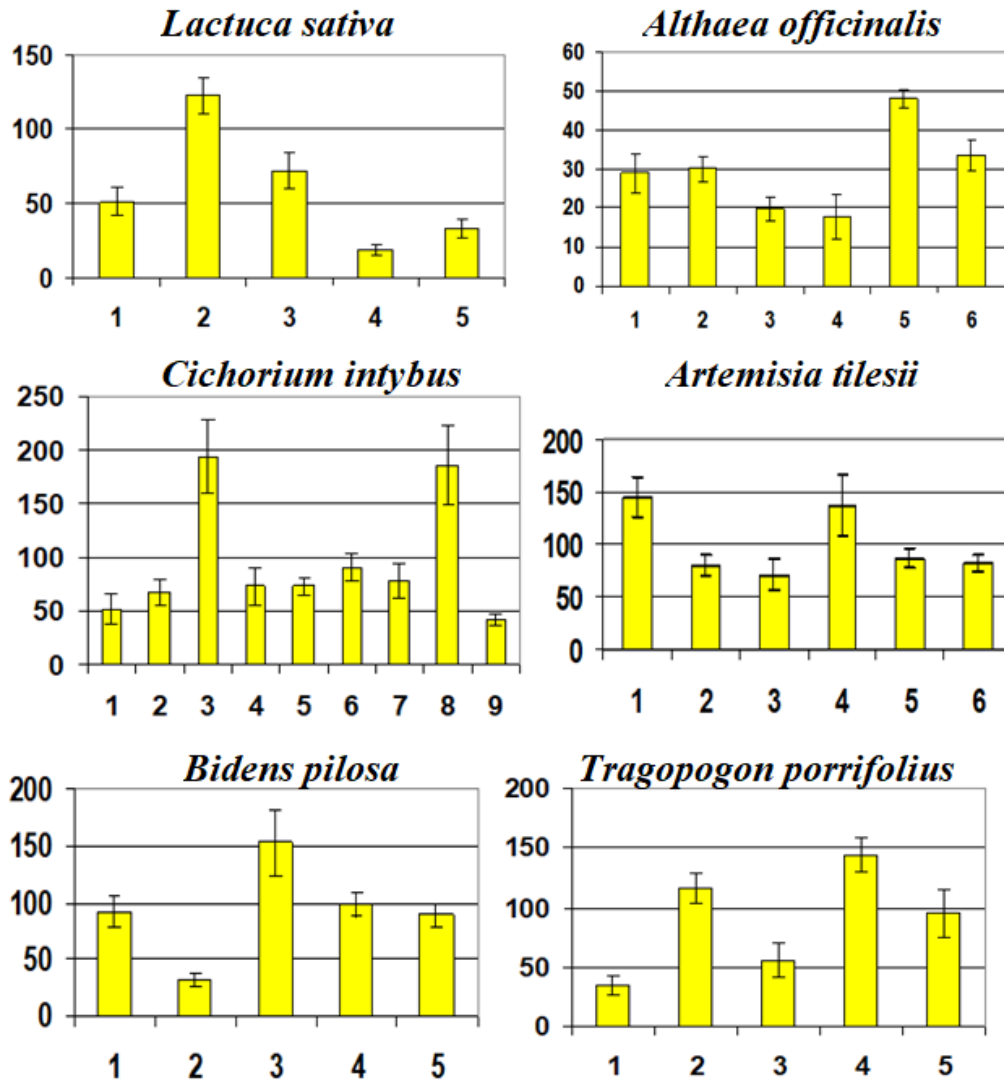

Figure 13 – Variations of fructan content (mg/g dry weight) in different plants: 1 – control; 2-9 – hairy root lines

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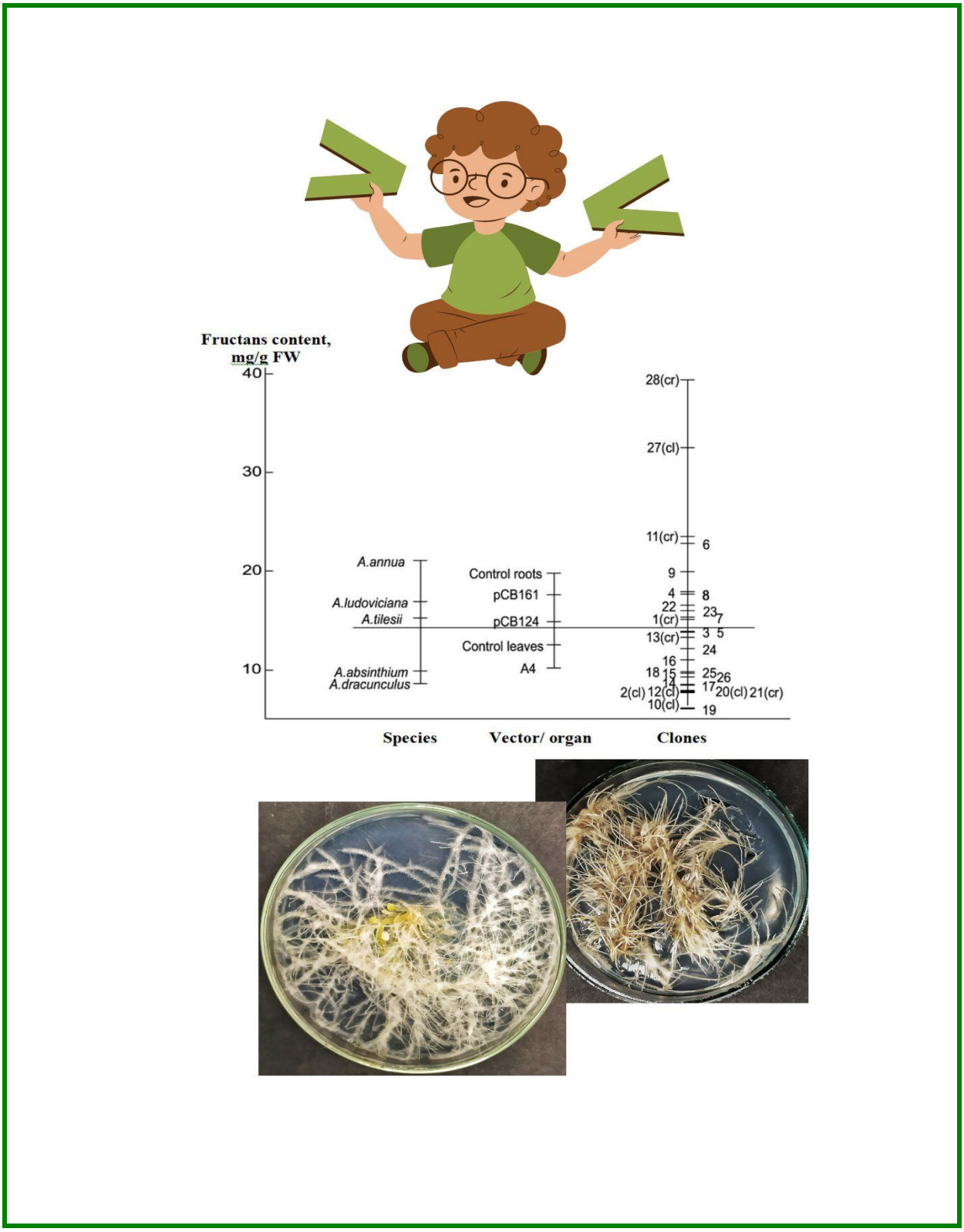

Figure 14 – Comparative assessment of fructans content in transgenic roots and control plants of *Artemisia annua, A. tilesii, A. dracunculus, A. ludoviciana,* and *A. absinthium*

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It is known that the addition of growth regulators to the medium in the case of *in vitro* cultivation of the samples can affect the growth of plants. Such addition can also affect the synthesis of various compounds in hairy roots, including fructans.

In our laboratory effect of some growth regulators Ivin, Emistim, Biolan, and Charkov ("AgroBioTech", Ukraine) on *Cichorium intybus* L. hairy root growth and fructans accumulation was studied (Tsyganкova et al., 2013) (Fig. 15). These regulators have a multi-component composition, which includes the products of a symbiotic myxomycete fungus isolated from ginseng roots (a mixture of amino acids, carbohydrates, fatty acids, polysaccharides, phytohormones, and microelements), which affect plant growth processes.

Every mentioned above regulator, added to the MS medium at a concentration of 2.5-10.0 µl/l stimulated root growth. Fructans content in hairy roots increased 7.0-35 fold and depended on the type of regulator and hairyroot line used in the experiment.

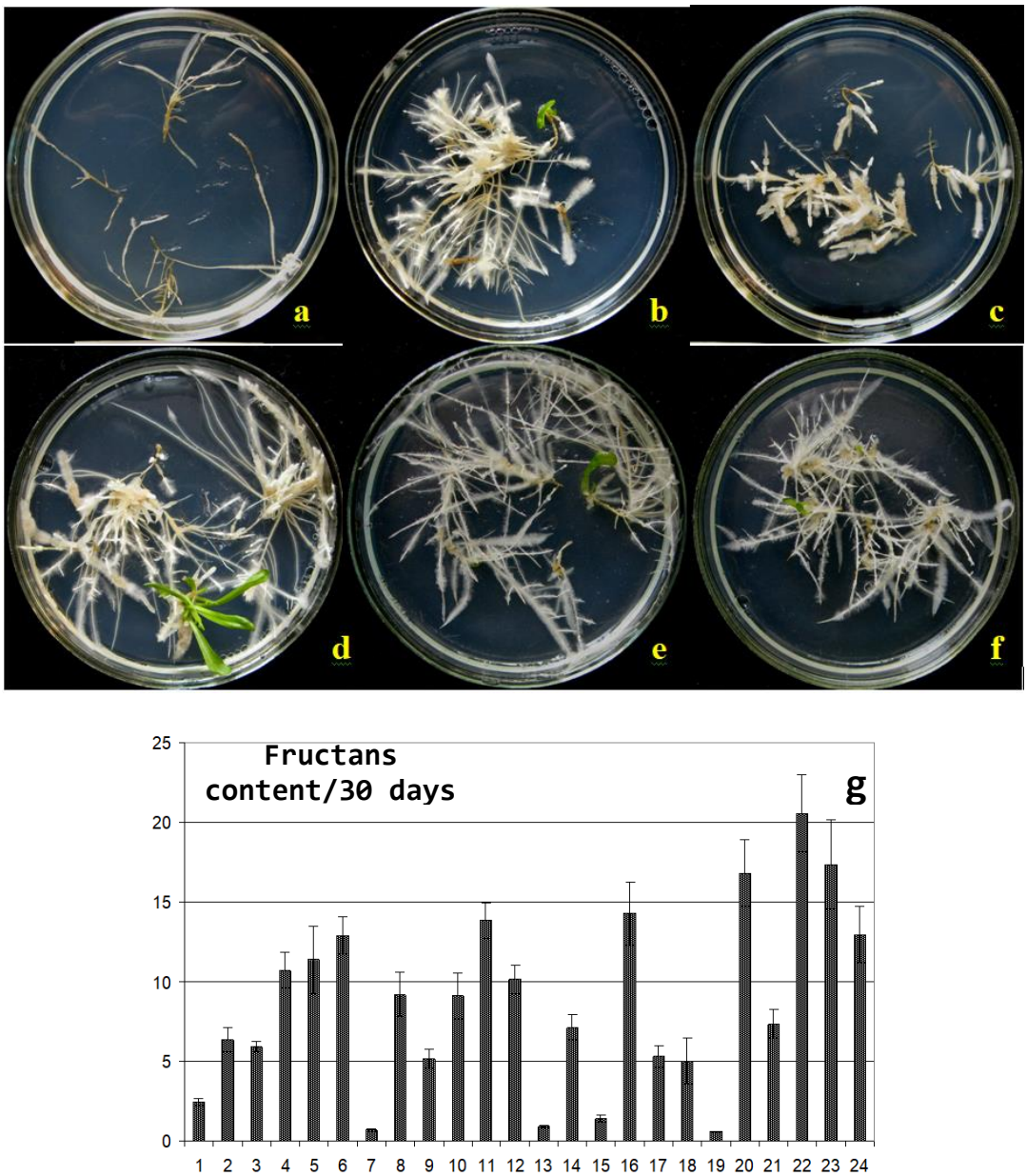

Figure 15 – Growth of chicory hairy root line on the medium supplemented with growth regulators ("AgroBioTech", Ukraine): Ivin (**b**, 2.5 µL/L; **c**, 5 µL/L) and Emistim (**d**, 2.5 µL/L; **e**, 5 µL/L; **f**, 10 µL/L), without regulators (**a**); **g** – total fructans content (1, 7, 13, 19 – without regulators)

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3.3. Flavonoid content

Flavonoids are a large group of polyphenolic compounds with a benzo-γ-pyrone structure synthesized by the phenylpropanoid pathway. These secondary metabolites can be found in different parts of plants. Flavonoids are associated with various biological effects (Dias et al., 2021) (Fig. 16). They are important compounds of plant cells that take part in the adaptation to stress factors (Agati et al., 2012). As a dietary component, flavonoids have health-promoting properties. Flavonoids are used in the food, cosmetic, and pharmaceutical industries (Kumar, Pandey, 2013b; Cortez et al., 2017; Khoo et al., 2017; Agrawal, 2011). Due to their bioactivity as potent antioxidants, flavonoids can be used for drug production (Cook, Samman, 1998; Miyake et al., 2000). It was reported the anti-inflammatory activity of flavonoids (Middleton, 1998; Murlidhar et al., 2010). They demonstrated antimicrobial (Górniak et al., 2019; Fathima et al., 2016; Xu et al., 2012) and antifungal activity (Redondo-Blanco et al., 2020; Al Aboody, Mickymaray, 2020).

Isoflavones, a group of flavonoids, are phytochemicals with potent estrogenic activity (Atkinson et al., 2004). In particular, such compounds as genistein, daidzein, and glycitein are accumulated in soybeans (Doerge, Sheehan, 2002). Their structure is similar to the human female hormone 17-β-estradiol, so they can be used for the treatment of some hormone-dependent diseases and in relieving postmenopausal symptoms. The clinical studies proved the possibility of genistein and daidzein use in the chemoprevention of breast and prostate cancer, cardiovascular disease, and osteoporosis (deVere White et al., 2010; Chen et al., 2005; Cassidy et al., 1996; Rodríguez-García et al., 2019). The same effect was found in the case of other flavonoids using (Ciumărnean et al., 2020; Ginwala et al., 2019; Zaidun et al., 2018; Tavsan et al., 2019; Zhang et al., 2017).

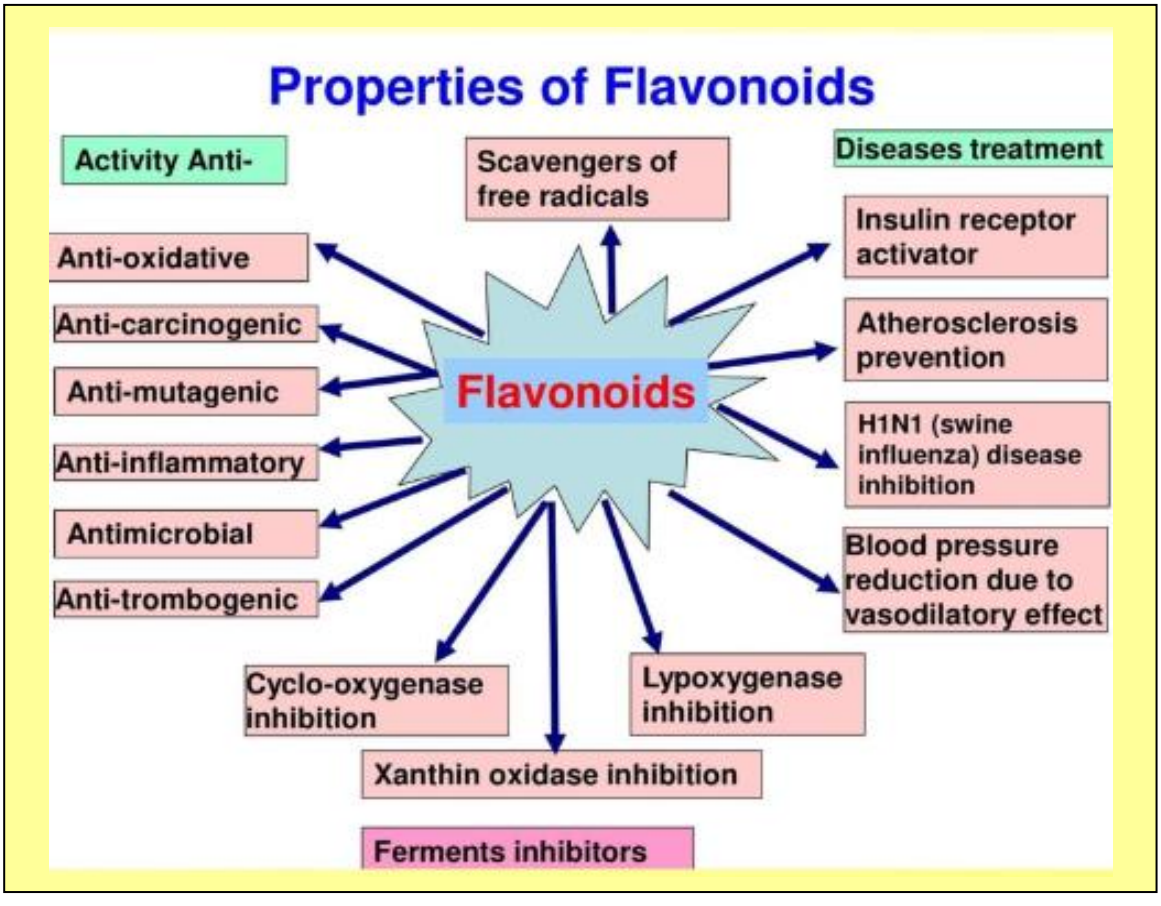

Figure 16 – Spectrum of bioactivity of plant-synthesized flavonoids

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We studied the content of flavonoids in different clones of hairy roots. This study reveals the significant difference between root clones and the increase of flavonoid content in some clones (Bohdanovych et al., 2021; Bohdanovych et al., 2021; Olkhovska and Matvieieva, 2021; Matvieieva et al., 2020; Kobylinska et al., 2020). For example, the high content of flavonoids was identified in *Artemisia vulgaris* hairy root clones. In particular, a 2.1 – 4.4 fold increase in flavonoid concentration was found. The same increase was studied in *A. dracunculus* (1.6 – 2.5 fold), *A. annua* (2.1-3.3 fold), and *A. tilesii* (1.1- 2.4 fold) hairyroot clones.

Four hairy root lines (No 2, 4, 10, and 16) of *A. tilesii* that differed in their morphology were used to compare flavonoid content (Fig. 17). They differed significantly in the growth rate and the content of flavonoids. The content of flavonoids in all these lines was higher than that in control roots (2.31 ± 0.42 mg RE/ g FW) and varied from 2.57 ± 0.28 mg RE/ g FW in line 4 to 9.47 ± 1.97 mg RE/ g FW in line 2. Such an increase in the content of flavonoids in extracts from hairyroots compared to non-transformed plants can be explained by an increase in the level of synthesis of secondary metabolites after the transfer of *rol* genes, because they are known as activators of metabolism in plants. Significant variability in the parameters between individual lines is probably caused by the non-determined incorporation of transferred genes into the plant genome. It is also important to note that no differences in flavonoid accumulation were observed between the two groups of lines. Hairy roots obtained as a result of transformation with a wild strain of agrobacteria (lines 10 and 16) and those containing *ifn-α2b* and *npt*II genes (lines 2 and 4) had approximately the same range of flavonoid concentration. Therefore, it can be concluded that these additional genes do not affect the biosynthesis of polyphenolic compounds (Bohdanovych et al., 2021).

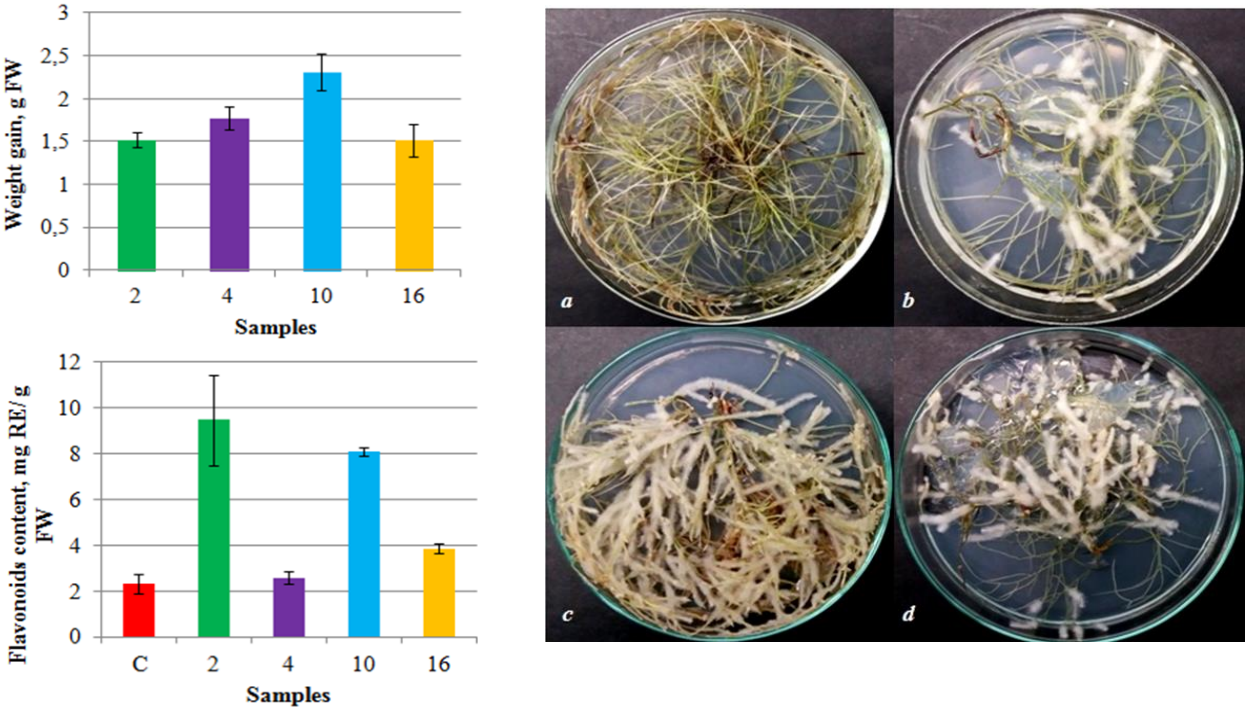

Figure 17 – *Artemisia tilesii* hairyroot lines and the differences in their growth and flavonoids accumulation: a – No 2; b – No 4; c – No 10; d – No 16 (Bohdanovych et al., 2022)

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3.4. Artemisinin and sugars production

We studied the effect of genetic transformation on the biologically active compound artemisinin and its co-products and sugars accumulation in *Artemisia vulgaris* and *Artemisia dracunculus* hairy root cultures.

The detection of artemisinin in hairy wormwood roots is noteworthy, as *Artemisia annua* plants are widely known as producers of this valuable metabolite. Artemisinin and its derivatives are sesquiterpene lactones with antimalarial activity (Xia et al., 2020). These plant-derived components are recommended as a drug for malaria-specific treatment. The secondary metabolites possess anti-inflammatory, antioxidant, and immunoregulatory properties (White et al., 2915; Wan et al., 2017; Shi et al., 2015; Roussel et al., 2017; Otuechere et al., 2012). The antiviral activity of artesunate was also detected (Sharma et al., 2014). Luo Y. et al. (2019) proposed artemisinin-based nanocomposition for specific cancer treatment.

In our study (Drobot et al., 2017), it was found that genetic transformation resulted in changes in artemisinin content in *A. vulgaris* hairy roots. Notably, the content was 0.237–1.02 mg/g dry weight in transformed roots (Drobot et al., 2016a). Therefore, the increase of artemisinin content up to 1.02 mg/g DW compared to the nontransformed roots (up to 0.687 mg/g DW) was observed (Fig. 18). In the case of both species, changes in artemisinin content in transgenic root lines did not depend on the vector used. Thus, *Agrobacterium rhizogenes*-mediated genetic transformation can be used for obtaining *A. vulgaris* and *A. dracunculus* hairy root culture produced artemisinin and sugars in a higher amount than mother plants (Fig. 18).

Glucose, fructose, sucrose, and mannitol were accumulated in *A. vulgaris* and *A. dracunculus* hairy root lines. In some cases, a genetic transformation has led to the sugar content increasing or appearing of nonrelevant for the control plant carbohydrates.

Control *A. vulgaris* and *A. dracunculus* plants cultivated in vitro differed in carbohydrate content. The content reached 61.7±0.21 and 14.235±0.24 mg/g DW, respectively. The high glucose content was also found in *A. dracunculus* leaves – 31.29±0.45 mg/g DW compared with 13.35±0.12 mg/g DW in the roots.

Fructose, glucose, and sucrose content was higher in the roots of *A. vulgaris* than in the leaves and reached 20.93±0.31, 17.37±0.26, and 25.48±1.14mg/g DW, respectively (14.71±0.28 mg/g, 10.15±0.1 mg/g and 12.27±1.39 mg/g DW in the leaves).

The transgenic roots not only differed in fructose, glucose, and sucrose content compared to the control but also accumulated nontypical for mother plant compounds. Sucrose content was 1.6 times higher in *A. vulgaris* hairy root lines than in the control roots. Fructose content was found to be 3.4 times higher in *A. dracunculus* hairy root cultures than in the control roots. The accumulation of galactose was a specific feature of the transgenic roots since this compound was not detected in the control nontransformed plants (Fig. 19).

Thus, the transformation affected the synthesis of metabolites of different groups - both artemisinin and sugars – in the transformed plant roots of the two plant species. Probably, this is the result of the transfer to the genome of plants of bacterial *rol* genes, known for their influence on the metabolism of plant cells. The detection of mannitol and galactose, which were absent in the leaves of the control plants is of special interest. This result suggests that the plants probably have genes encoding the synthesis of these compounds, but the genes are usually inactive in the leaves. At the same time, the genetic transformation and incorporation of bacterial genes into the plant genome led to their activation resulting in the appearance of mannitol and galactose in hairy roots.

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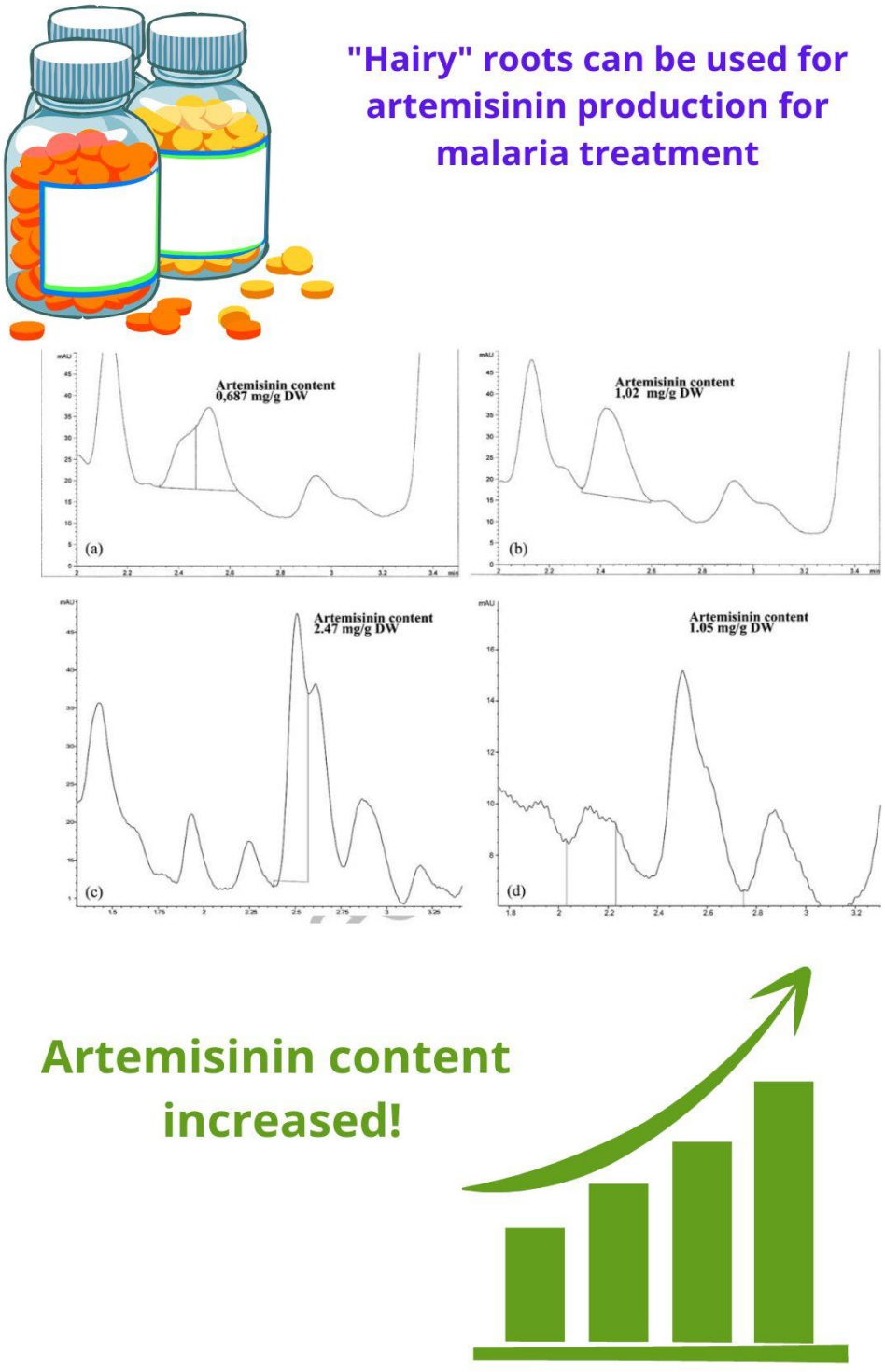

Figure 18 – HPLC traces of artemisinin content obtained for roots of control *Artemisia vulgaris* plants (a); *A. vulgaris* hairy roots (b); *A. dracunculus* control roots (c); *A. dracunculus* hairy roots (d) (Drobot et al., 2017)

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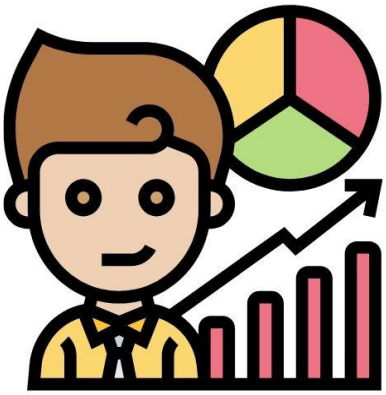

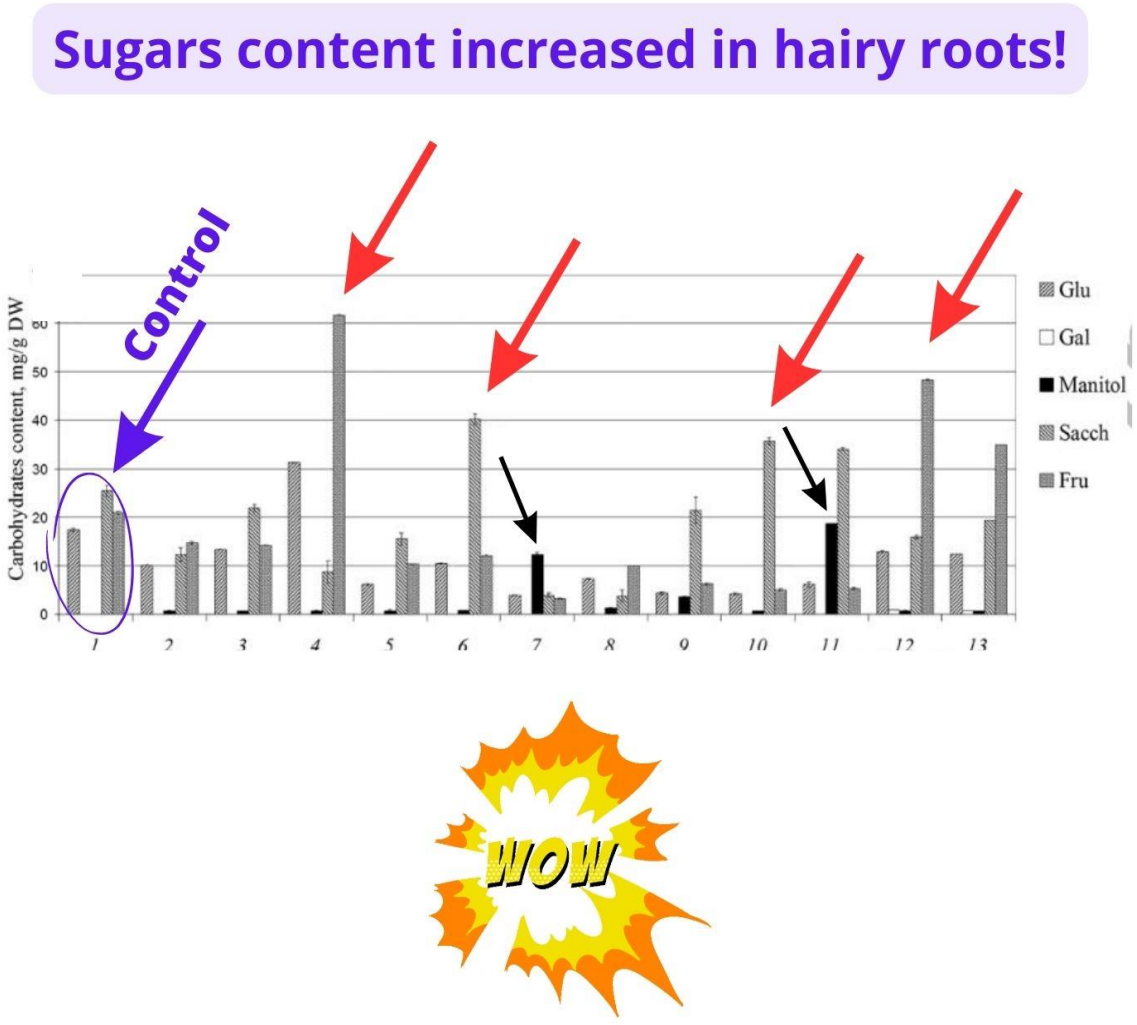

Figure 19 – Sugars content (Glu – glucose, Gal – galactose, Mann – mannitol, Sucr – sucrose and Fru – fructose) in *Artemisia vulgaris* non-transformed plants (1 – leaves, 2 – roots) and hairy root transgenic lines (5–9).

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3.5. Variations of antioxidant activity

The system of antioxidant protection of plants is very complex. In a simplified way, it can be represented as the activity of antioxidant defense enzymes (primarily superoxide dismutase, catalase, peroxidase), as well as the activity of some plant metabolites, in particular, polyphenols, the synthesis of which can be inhibited or increased under certain conditions (for example, under the influence of stress factors of various origins). Transgenic roots differ from mother (nontransformed) plants by the presence of different transferred genes. Can these foreign genes affect the activity of the antioxidant defense system in hairy roots? We studied this problem using transgenic roots of different plant species.

The effect of *A. rhizogenes*-mediated transformation on the antioxidant status of *Artemisia tilesii, A. vulgaris, A. dracunculus*, and *A. annua* transgenic roots has been studied (Matvieieva et al., 2018a). Antioxidant activity (AOA) of aqueous extracts was determined using methods based on the ability to reduce DPPH and ABTS radicals. The level of AOA (DPPH) in 50% of extracts obtained from transgenic roots was higher than the level of activity possessed by extracts from untransformed roots. An increased ability to reduce the ABTS+ radical was observed in 80% of the extracts. Extracts of *A. annua* and *A. tilesii* transgenic roots were the most active, while the lowest antioxidant activity was shown in *A. dracunculus* extracts. Thus, *A. rhizogenes*-mediated transformation has changed the antioxidant status of the hairy roots of several *Artemisia* spp. plants (except *A. vulgaris*).

In another study (Bohdanovych et al., 2022) the correlation between antioxidant activity (DPPH test) and total flavonoid content was evaluated. The total content of flavonoids and the levels of antioxidant activity in *Artemisia tilesii* hairy root lines with different transferred genes were compared. The content of flavonoids in most of the lines was higher than in the control plants and correlated with antioxidant activity (Fig. 20, 21, 24). The higher content of flavonoids corresponds with the higher antioxidant activity.

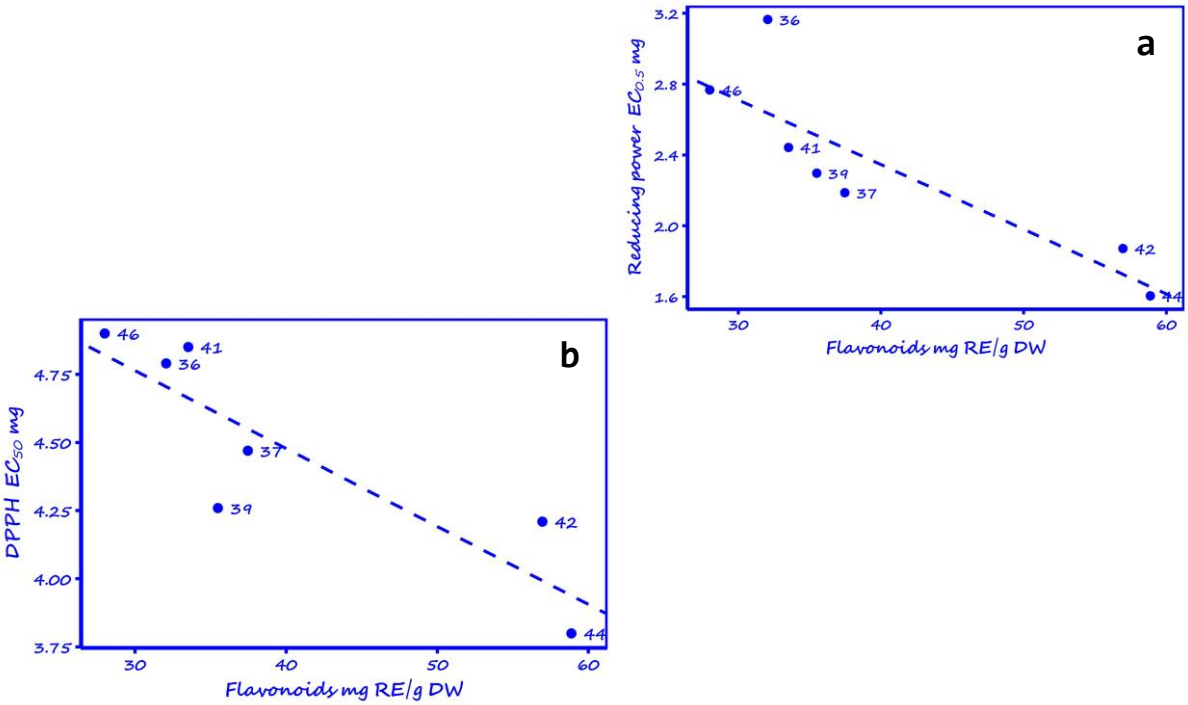

Figure 20 – Strong correlation between flavonoid content and reducing power (a) or flavonoid content and antioxidant activity (b) in the samples of *Artemisia tilesii* hairyroots (Matvieieva et al., 2020)

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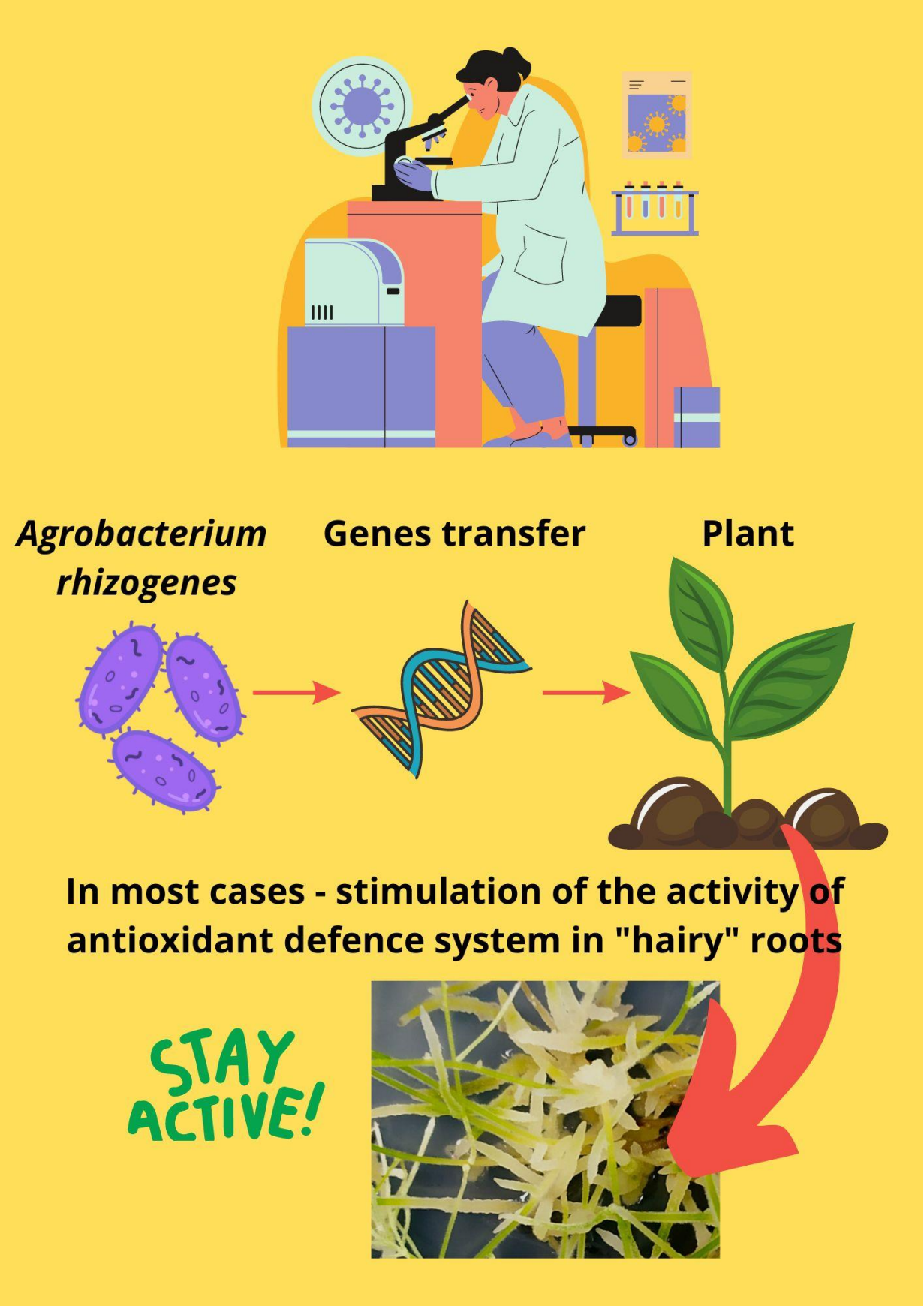

Figure 21 – Genetic transformation affected the activity of the antioxidant defense system in hairy roots

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The activity of one of the key enzymes of the antioxidant defense system – superoxide dismutase (SOD) – was determined in *Artemisia* spp. and *Althaea officinalis* hairy root lines (Matvieieva et al., 2022a) (Fig. 22) obtained after the transformation by wild A4 *Agrobacterium rhizogenes* strain.

SOD activity in the roots of untransformed *in vitro* cultivated plants used for the initiation of hairy roots growth was in the range from 45.8 ± 8.7 U/μg (*A. officinalis*) to 275 ± 97.1 U/μg (*A. ludoviciana*). More than half of tested hairy root lines (54%) showed a significant increase in SOD activity compared to untransformed roots. The highest SOD activity values of hairy root extracts (24-fold increase) were founded for *A. officinalis* (1105 ± 174 U/μg) and *A. dracunculus* (1356 ± 402 U/μg). The increased SOD was also found in the hairy roots of *A. vulgaris* (up to 375 ± 28.2 U/μg, sevenfold increase), *A. ludoviciana* (1001 ± 191 U/μg, 3.6-fold increase), and *A. tilesii* (438 ± 104 U/μg, 1.6-fold increase). The results of our study indicated that transformation by wild-type *A. rhizogenes* not harboring any foreign genes implemented in SOD activity regulation can stably activate the plant antioxidant enzyme system (Fig. 23).

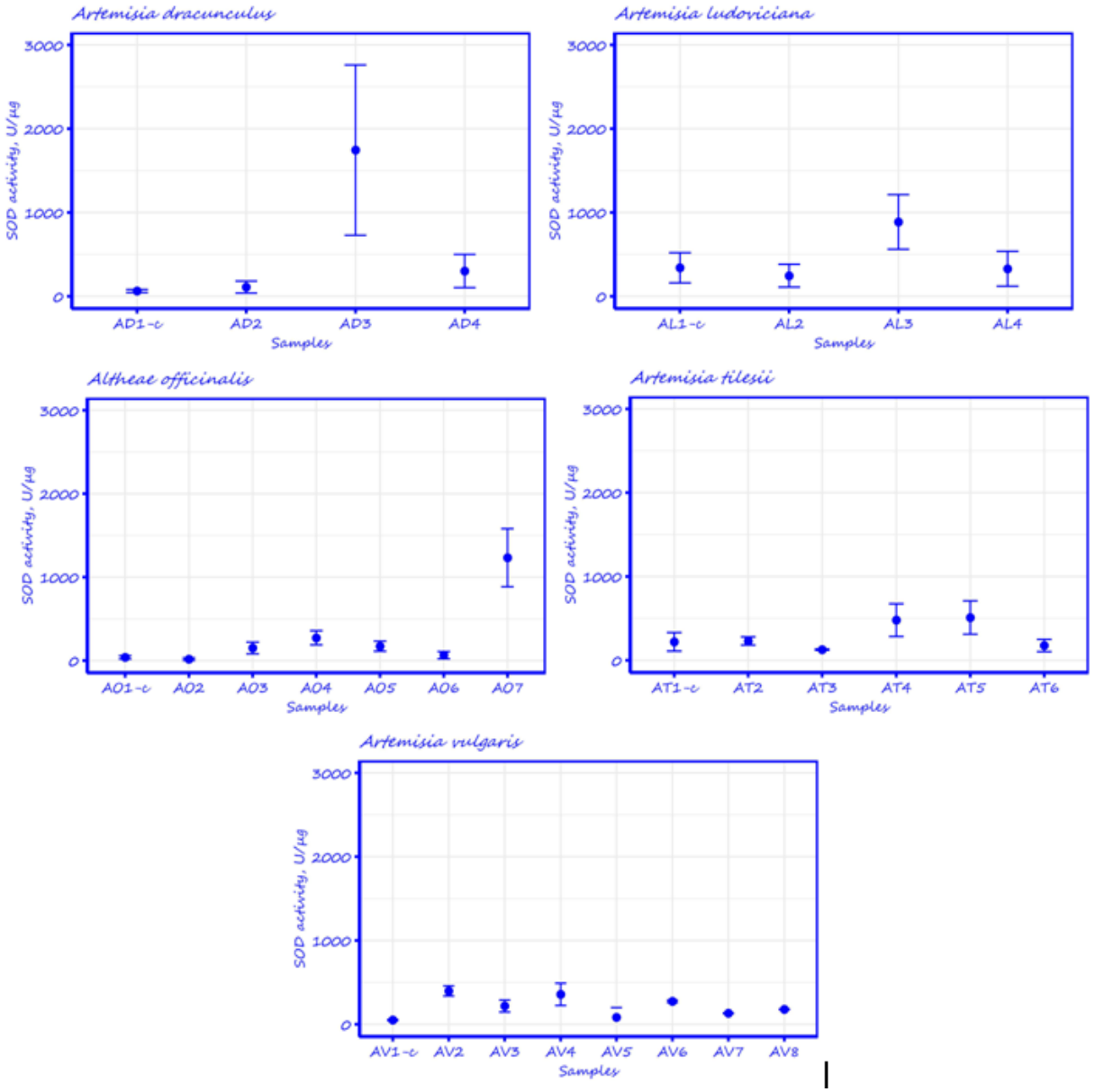

Figure 22 – Superoxide dismutase activity in hairy root clones of different plant species (Matvieieva et al., 2022a)

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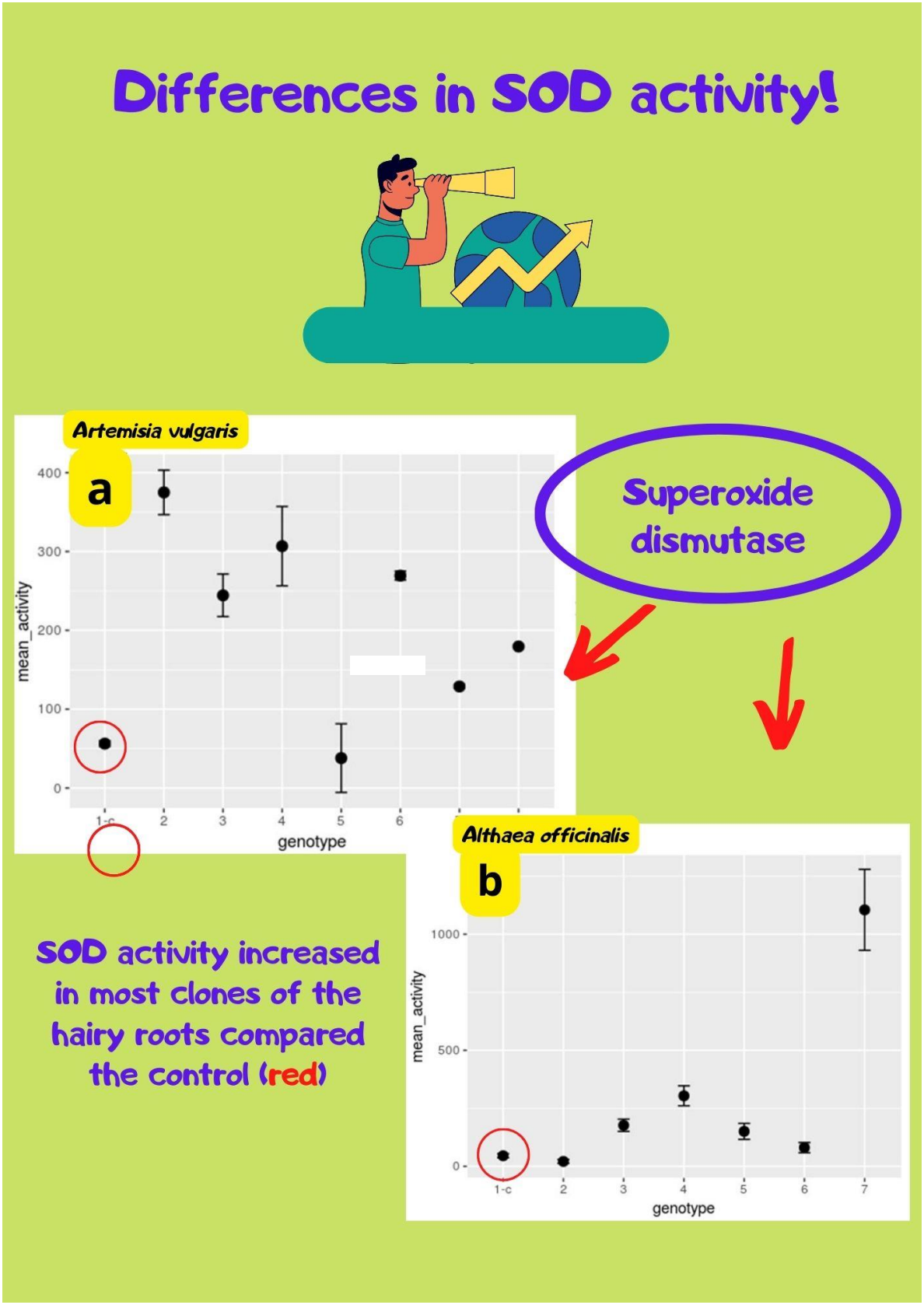

Figure 23 – Differences in superoxide dismutase activity in hairyroot clones (a – *Artemisia vulgaris*, b – *Althaea officinalis*) can be initiated by the genetic transformation

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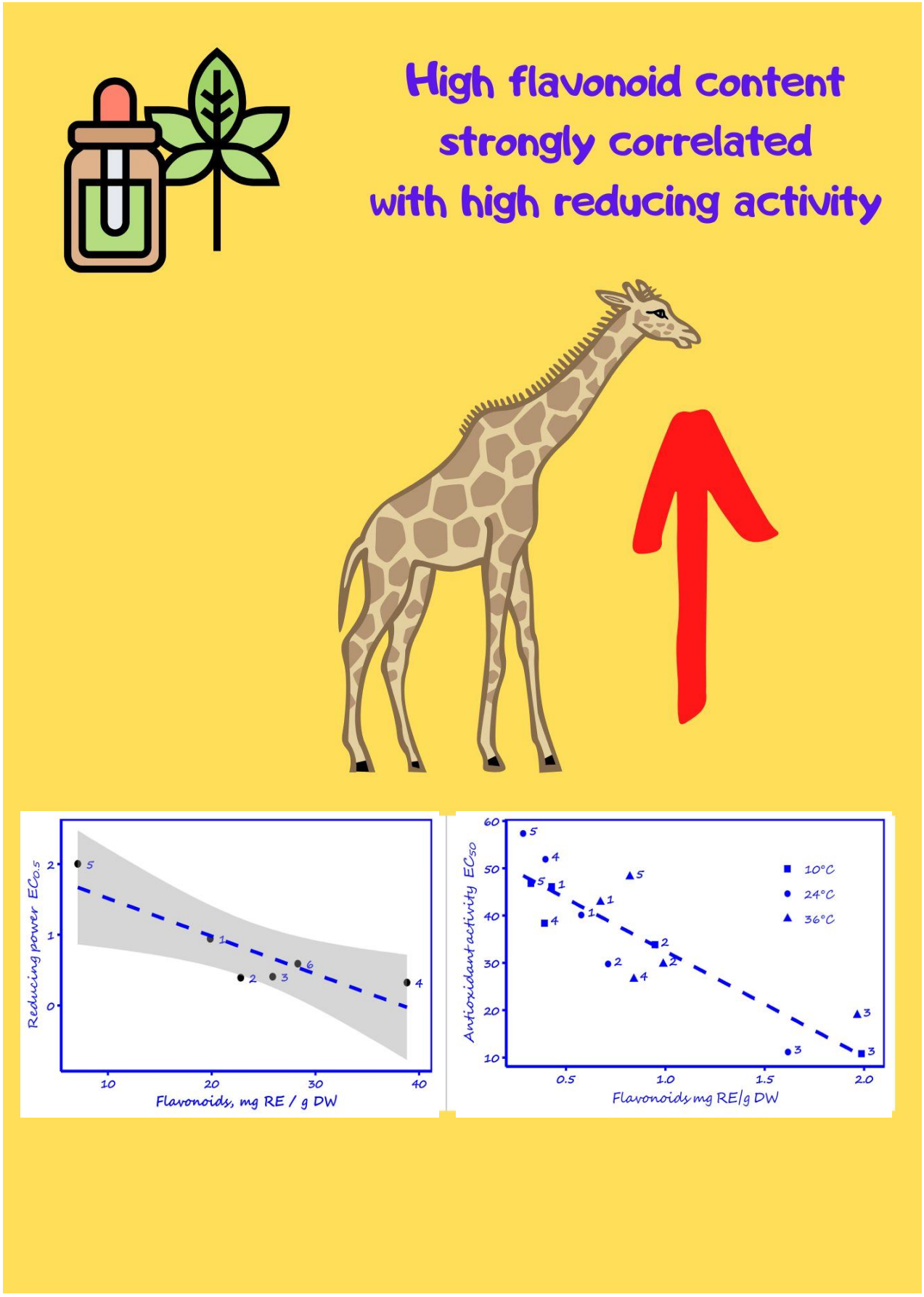

Figure 24 – Correlation between flavonoid content in the extracts of hairy roots and their reducing (Kobylinska et al., 2021) or antioxidant activity (Matvieieva et al., 2020).

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3.6. Effect of stress factors on growth and biosynthetic activity of hairy roots

The short-term high-temperature treatment affected the flavonoid accumulation in *Artemisia vulgaris* L. and *A. dracunculus* L hairy roots (Matvieieva et al., 2018b). The roots were cultivated for one, two, and five days at +36 °C, then were grown at +24°C for up to four weeks. Hairy root lines differed in their sensitivity to short-term high-temperature exposure. Both stimulation and inhibition of flavonoid accumulation, as well as no changes, were observed. A significant (1.7–6.4 times) decrease in the flavonoid content was observed in lines that showed higher flavonoid content under standard conditions (+24° C) without the temperature stress exposure.

In addition, we compared a postponed response of hairy roots (*Althaea officinalis* L.) of short-term cold- (+10°, 7 days) and high-temperature (+36°, 7 days) stresses factors (Matvieieva et al., 2021a). The results have shown a wide range of sensitivities to short-term temperature stress. This effect depended on the root line but was independent of the transformation vectors used for the transformation and the presence of the human *ifn*-α2b gene. It may be explained by the random insertion of transferred DNA in individual transformational events.

High temperature caused significant growth inhibition of all root lines, except those, which had the highest flavonoid content under the control conditions (Fig. 25, 26). Short-term cultivation of hairy roots at a low temperature did not cause high growth suppression, especially in the case of root line No2. This sample was resistant to the short-term effect of low temperature. In parallel with growth inhibition caused by a temperature increase, the activation of flavonoid synthesis as a response to this stress factor was observed. The study has shown a strong (R^2 = 0.78) linear dependence between the antioxidant activity of extracts from hairy roots and their flavonoid content. Thus, flavonoids participate in the response and adaptation of hairy roots to high-temperature stress (Matvieieva et al., 2021a).

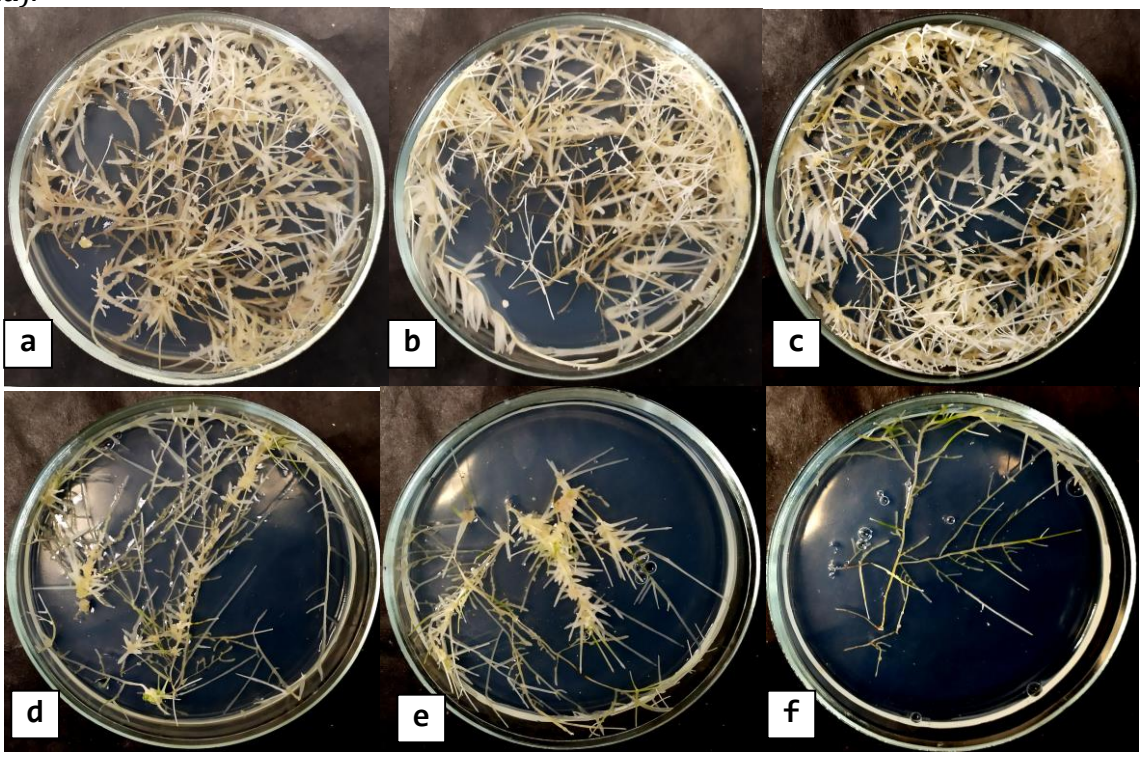

Figure 25 – Growth of hairy roots of *Althaea officinalis*, lines no 2 (a, b, c) and no 3 (d, e, f): a, d – growth under control conditions; b, e – root growth after short-term cultivation at +10°C; c, f – root growth after short-term cultivation at +36°C (Matvieieva et al., 2021a)

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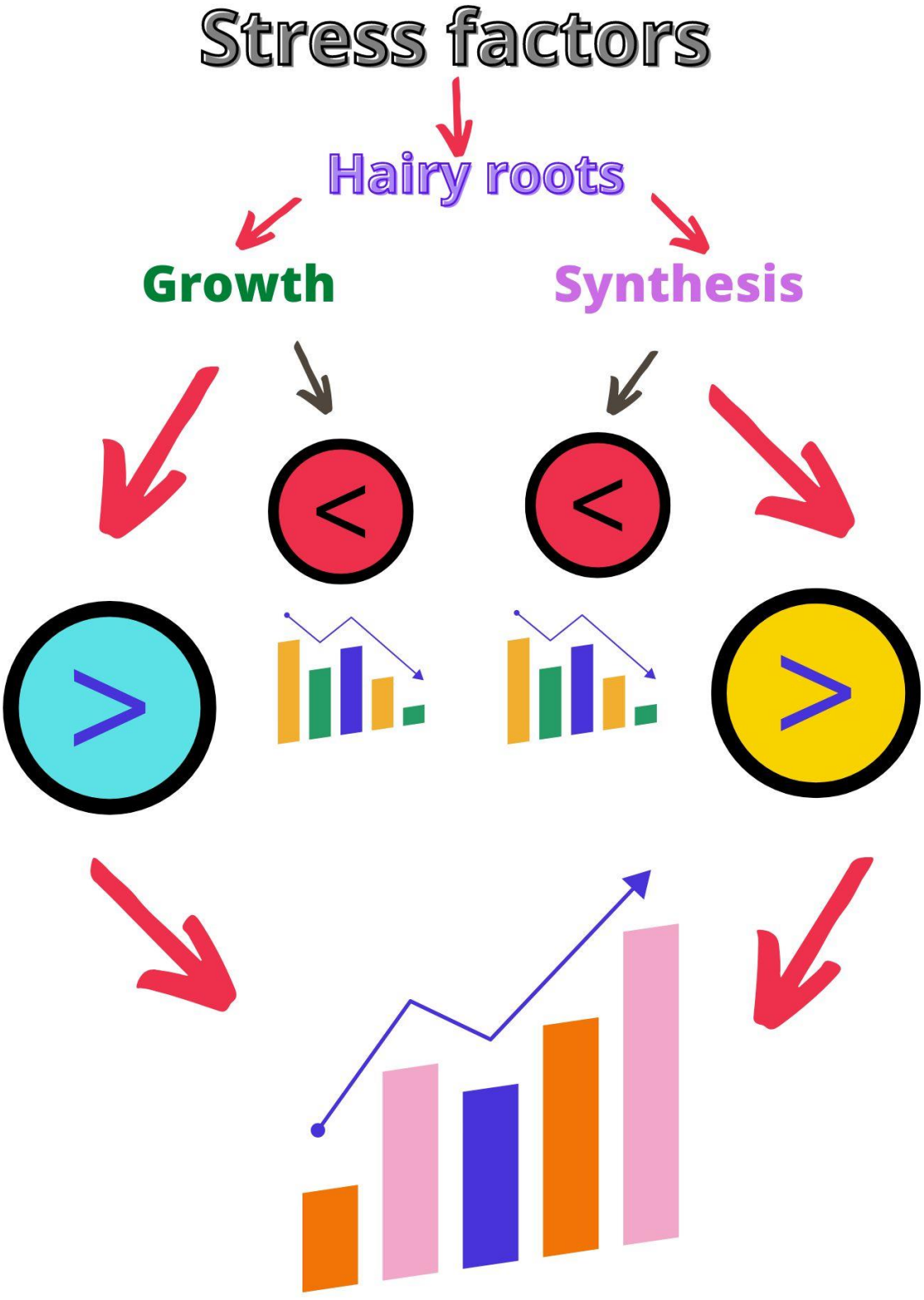

Figure 26 – Stress factors of different origins can affect growth rate and the biosynthetic activity of hairy roots

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3.7. Effect of nitrogen content on the biosynthetic activity of hairy roots

The content of nitrogen compounds in the soil or nutrient medium can alter the growth of plants, root cultures, and cells, both *in vivo* and *in vitro*. However, genetic transformation using soil phytopathogenic bacteria *Agrobacterium rhizogenes* can lead to changes in the functioning of plant cells, their ability to adapt to growing conditions, and biosynthetic activity. We studied the effects of reducing the content of nitrate salts in the nutrient medium on the growth of hairy roots of *Cichorium intybus* L. as a model (Matvieieva et al., 2021b).

Two root lines earlier obtained using the genetic transformation of chicory plants by *A. rhizogenes* A4 were studied. The roots were cultured on solidified Murashige and Skoog nutrient medium with standard (MS) and reduced nitrate salts content.

Differences in the growth rate of the roots of the two lines were revealed (Fig. 27). One line (5/1) was sensitive to such change of the medium that was expressed in a decrease in weight gain. However, the growth of the hairy roots of the second line (5/2) on the modified medium did not differ significantly from the control.

It should be noted that a decrease in the total content of flavonoids, as well as in antioxidant and reducing activities was observed in both samples. Such differences probably are related to the peculiarities of the genetic transformation of plant DNA using agrobacteria, in which the site of incorporation of transferred genes is indeterminate.

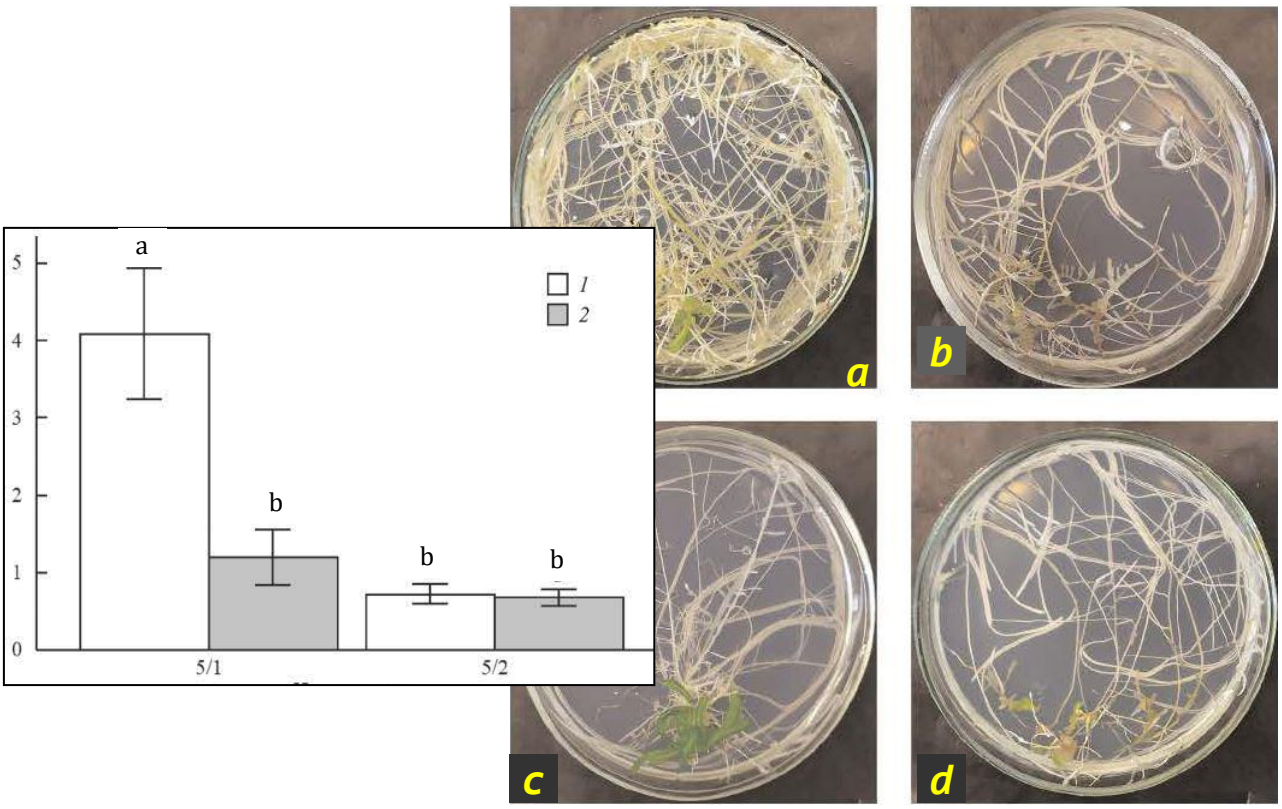

Figure 27 – Differences in the effect of Nitrogen content (in nitrate form) on the growth rate of two hairy root lines of chicory (Matvieieva et al., 2021b): **1** and ***a***, ***c*** – control MS medium was used; **2** and ***b***, ***d*** – the medium with a twice reduced nitrates content was used

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3.8. Effect of toxic metal on the growth and biosynthetic activity of hairy roots

The roots were able to interact with vanadium(IV) compounds. The compound turned out to be toxic, which was manifested in a decrease in the weight of the roots on the medium with vanadium (Fig. 28). Since vanadates are stereochemical analogs of phosphate, it can be assumed that the compounds entered the cells using the phosphate transport system.

The effect of toxic vanadium(IV) was studied by cultivating *Artemisia tilesii* hairy roots in the medium in concentrations from 1 to 100 mg/l. Hairy roots were more resistant to toxic metal than the mother plants. The addition of 5 mg/l of V(IV) to the MS medium led to complete inhibition of plant growth. At the same time, hairy roots survived on a medium containing 100 mg/l of V(IV). An increase in the concentration of vanadium(IV) in the medium from 1 to 100 mg/l has led to a significant increase in the content of flavonoids (by 35%) in the hairy roots. The antioxidant activity of the extract from the roots also increased under the influence of vanadium(IV) in concentrations of 1 and 5 mg/l by 11% and 20%.

Absorption of V(IV) by transgenic roots and its accumulation occurred both at its minimum (1 mg/l) and maximum (100 mg/l) concentrations in the culture medium. Quite expectedly, the maximum amount of V(IV), 80 μg/g of root weight, was accumulated by the roots growing at 100 mg/l of V(IV), and the minimum amount when growing at 1 mg/l (11 μg/g of root weight). However, with an increase in the concentration of toxic vanadium (IV), the efficiency of its absorption from the nutrient medium decreased significantly. The highest efficiency of V(IV) removal by hairy roots was observed at a concentration of 1 mg/l in the medium and reached 60%. The lowest immobilization efficiency (3%) was observed at a concentration of 100 mg/l.

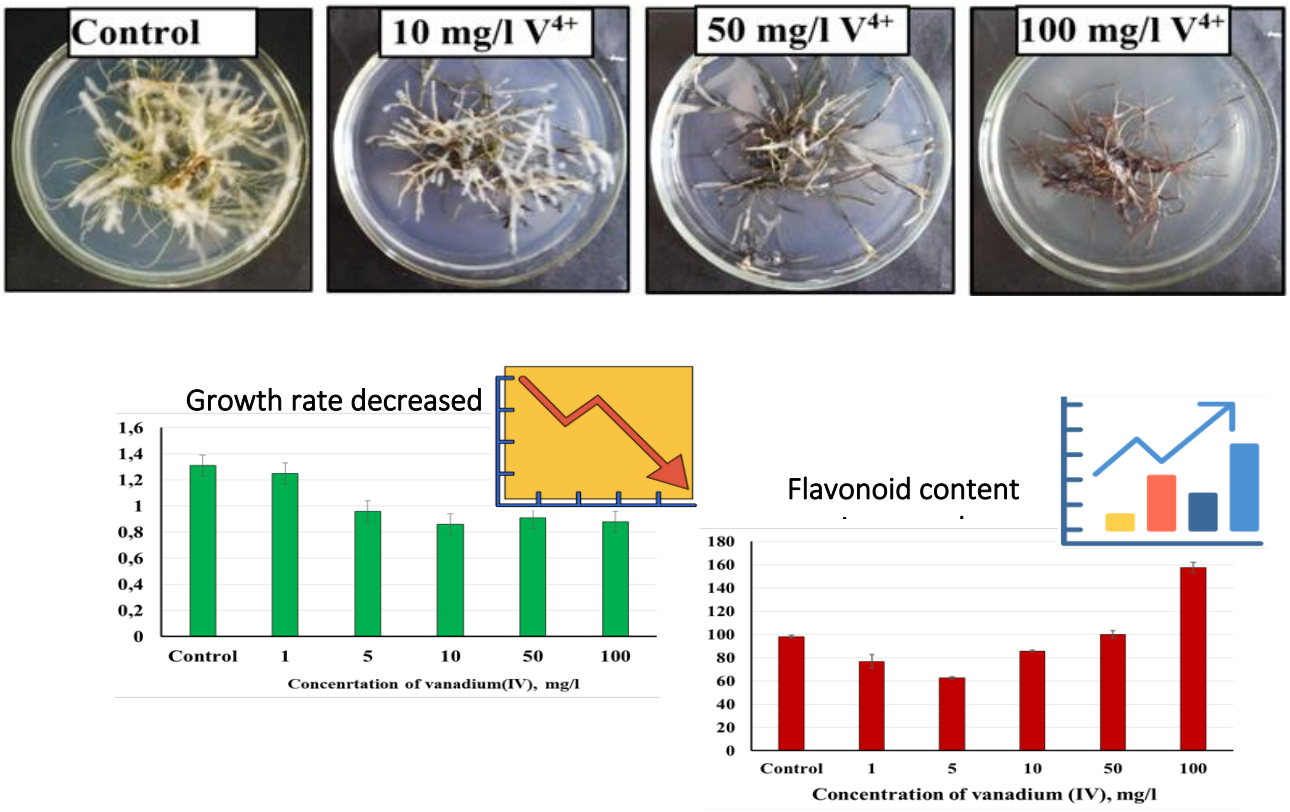

Figure 28 – Effect of adding toxic metal to the medium on growth rate and flavonoid content of *Artemisia tilesii* hairy roots

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3.9. Effect of sucrose content on growth of hairy roots

Carbohydrates are the compounds that participate in plant growth and the synthesis of metabolites. This is because these chemicals are the source of Carbon and energy for the plants. When plants grow *in vitro*, optimal concentrations of sugars that promote growth and biosynthesis must be determined for each plant species.

The influence of the concentration of sugars in the nutrient medium on the growth of roots was determined (Bohdanovych et al., 2021). For this, three variants of the 1/2MS medium were used: with the addition of 30 g/l of sucrose, with the content of sucrose reduced to 20 g/l, and with the addition of 20 g/l of sucrose and 10 g/l of fructose as carbon sources.

It was stated that the most increase in root weight was at a medium with sucrose at a concentration of 30 g/l (Fig. 29, 31). Reducing the sucrose content to 20 g/l led to a decrease in root weight by 17.4% and, in the case of sample cultivation on the same medium supplemented with fructose, root weight decreased by 15.4%.

Although the root growth rate was lower when the sucrose content in the medium was increased up to 40 g/l, and the specific content of flavonoids was higher under such conditions.

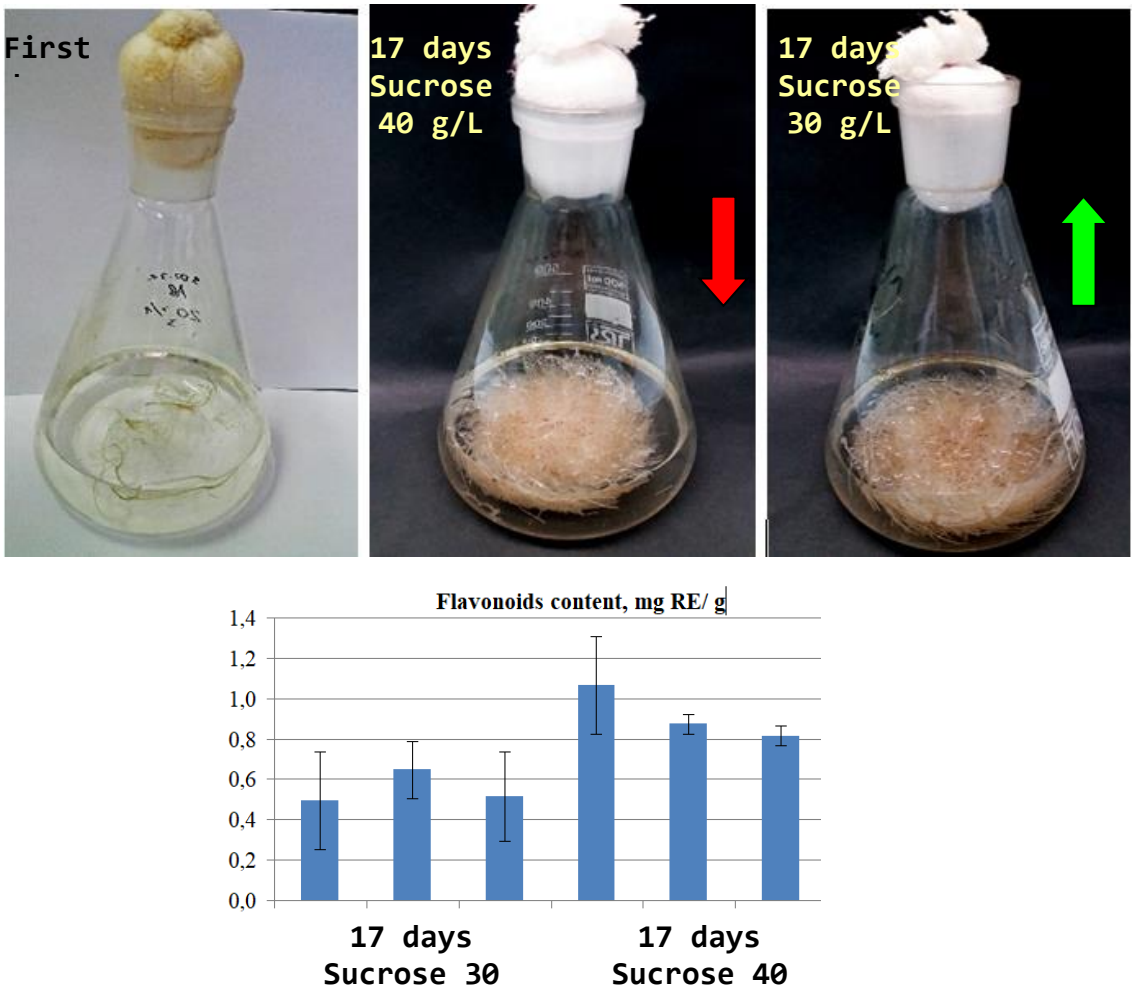

Figure 29 – Growth of hairy roots and flavonoid accumulation in the medium with different sucrose content

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3.10. Effect of compounds synthesized by bacteria on hairy roots growth

The interaction between soil microorganisms and plants has attracted the attention of researchers for many years. Since microorganisms in close contact with the rhizosphere can affect the vital activity of plants, the yield of crops may reduce or increase. At the same time, the factor of the direct effect of compounds excreted by soil bacteria, which are known for their positive influence on plants, particularly on their growth and biosynthetic activity, remains understudied. We proposed the model where the sterile culture liquid (test solution) obtained after the one-day cultivation of the *Priestia endophytica* UKM B-7515 bacteria interacted with the hairy roots of the wormwood (*Artemisia tilesii*) (Matvieieva et al., 2022b).

The roots of the two lines differed in sensitivity to the test solution by the growth rate. An increase in the concentration of the test solution in the nutrient medium stimulated the growth of root line No10. Adding 0.025, 0.05, and 0.1% of the test solution increased the root weight by 1.69, 2.31, and 2.54 times respectively. At the same time, no differences were found in the growth of the root weight of line No4 (Fig. 30, 31).

Therefore, a free of bacterial cells culture medium obtained after one-day cultivation of *Priestia endophytica* UKM B-7515 bacteria can stimulate the growth of some hairy root cultures and not affect others.

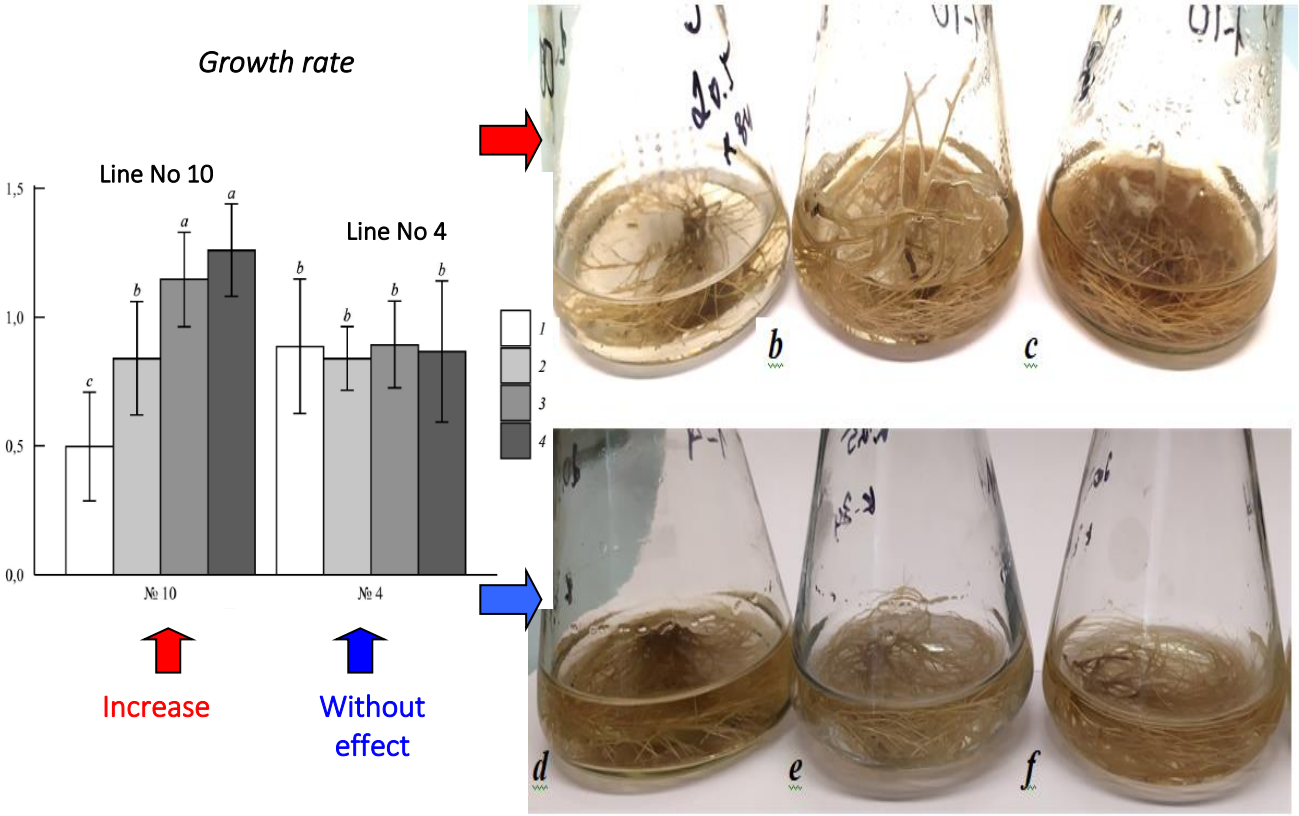

Figure 30 – Cultivation of roots of *Artemisia tilesii* root lines No. 10 (***a, b, c***) and No. 4 (***d, e, f***) in 1/2MS nutrient medium with the addition of a test solution at a concentration of 0 (***a, d***), 0.025 (***b, e***), and 0.1% (***c, f***) (Matvieieva et al., 2022b).

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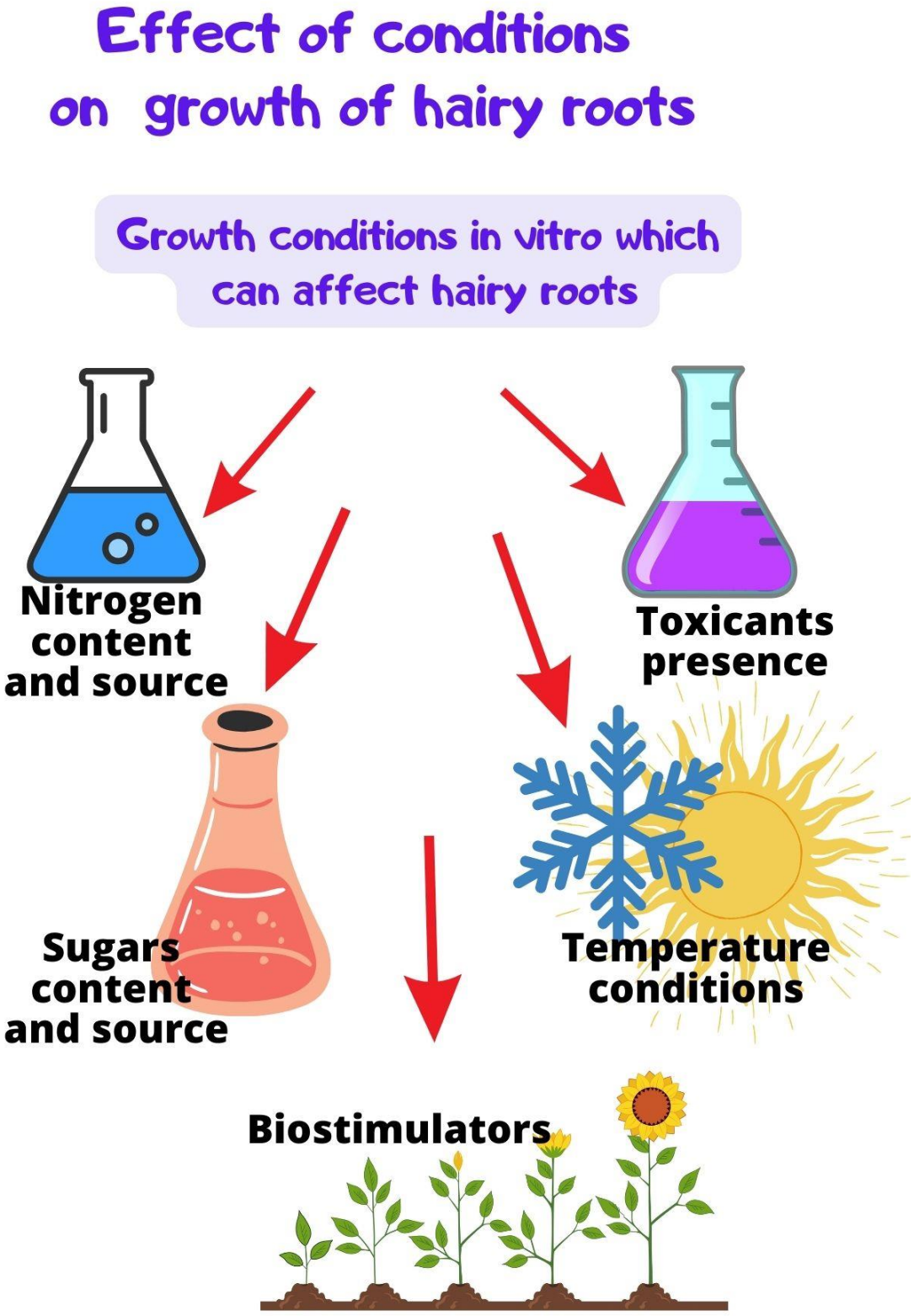

Figure 31 – Different factors that can affect hairy root growth parameters and bioactivity

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3.11. Antiviral activity of the extracts of hairy roots

The determination of the presence or absence of antiviral activity of extracts from the hairy roots of plants of various species was the next part of the work of the Laboratory of adaptation biotechnology ICBGE NAS of Ukraine. In our work, we had the opportunity to use both agrobacteria carrying plasmids with a human interferon-α2b gene and a wild strain for plant genetic transformation. The human interferon-α2b gene can improve antiviral activity under the suitable expression conditions of the gene and the synthesis of the corresponding protein. Plants produced recombinant proteins can be used for the synthesis of valuable compounds for further extraction or without extraction as a so-called "edible" vaccine that provides direct human or animal immunization (Walmsley et al., 2003; Joensuu et al., 2008; Gunasekaran & Gothandam, 2020).

We transferred human interferon-α2b gene (*ifn*-α2b) in *Cichorium intybus, Artemisia tilesii*, *Bidens pilosa, Althaea officinalis,* and *Lactuca sativa* (Matvieieva et al., 2009; Matvieieva et al., 2012a). The antiviral activity of extracts from transgenic plants and roots was studied (Matvieieva et al., 2012b).

The antiviral activity of extracts from different transgenic root lines of each species (*B. pilosa, A. tilesii, A. officinalis, L. sativa, C. intybus*) significantly differed. This difference may be explained by the fact that each transgenic line is an independent transformational event. The level of antiviral activity of the extracts varied in transgenic root lines of different plant species studied in our work. The high antiviral activity tested on the MDBK cell line was identified in extracts of *L. sativa* (up to 14062 IU/g) and *A. officinalis* (up to 40760 IU/g). The highest antiviral activity was found in extracts of *A. tilesii* transgenic roots – up to 98437 IU/g (Fig. 32, 33).

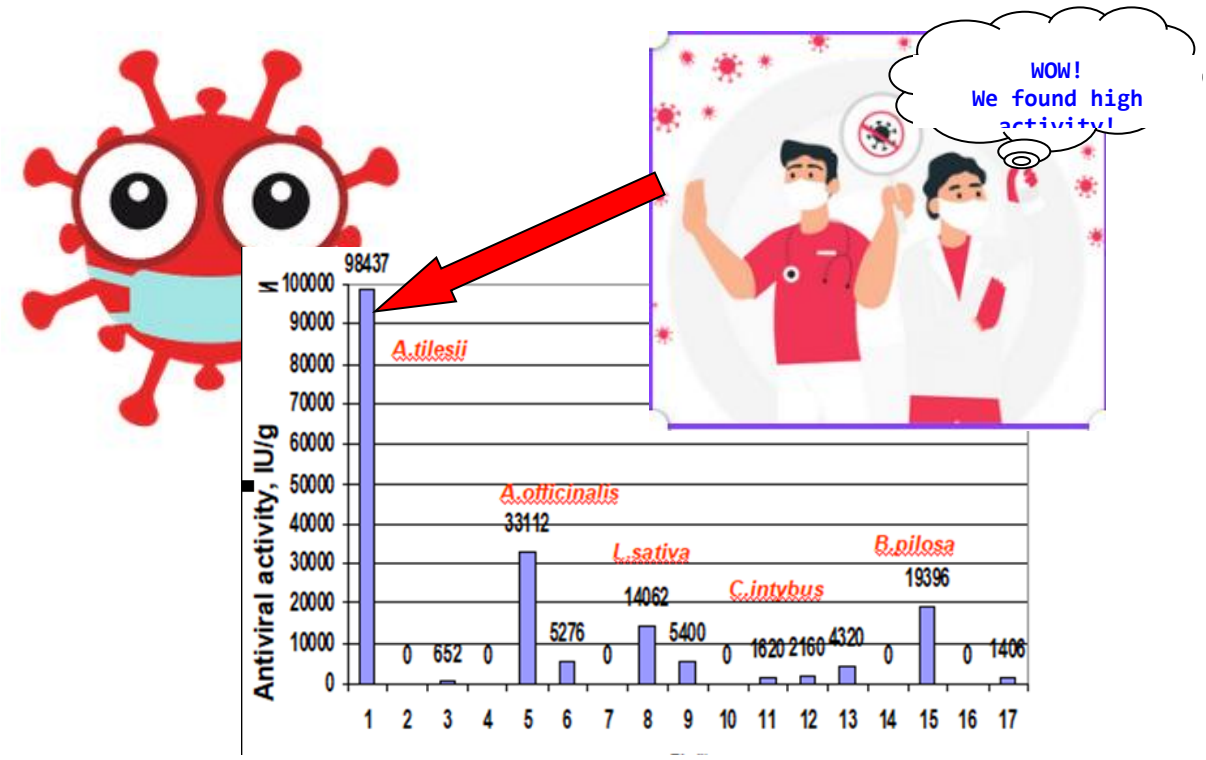

Figure 32 – Antiviral activity of the extracts of hairy roots of different plant species

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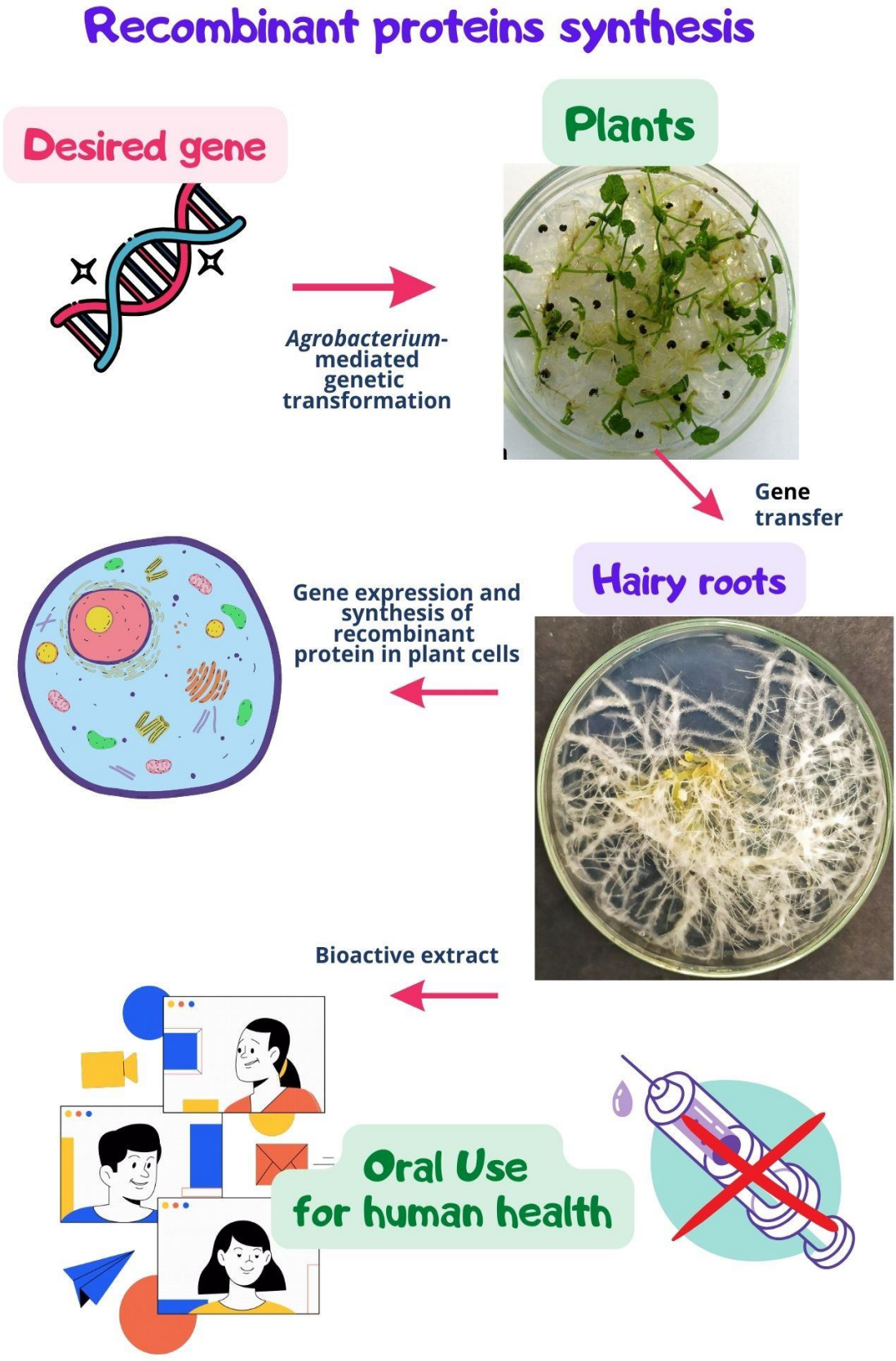

Figure 33 – Potential use of recombinant proteins synthesized in hairy roots

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3.12. The DNA-protective activity of hairy root extracts

DNA damage is one of the significant negative effects of free radicals and oxidative stress. Reactive oxygen species damage DNA by strand breaks and base oxidation resulting in modifications of DNA structure. Such a modification, in turn, leads to changes in coding and, accordingly, to a violation of the essential functions of DNA in cells. Such a process can leads to apoptosis of the cells and the initiation of cancer**.** That is why the search for new compounds that can effectively protect DNA from ROS-induced damage continues.

It was studied that some plant-synthesized chemicals can protect DNA from oxidative stress. Flavonoids were identified as effective DNA protectors. For example, Min et al. (2009) proved quercetin as an inhibitor of hydrogen peroxide-induced DNA damage. The flavonoids also enhance DNA repair. Polyphenols protect DNA from oxidative damage (Silva et al., 2008). Quercetin was studied for cancer prevention due to its antioxidant activity (Vargas et al., 2010). Rich in flavonoids Kiwifruit was recommended as a modulator of DNA damage and DNA repair (Collins, 2013).

DNA-protective activity can be studied *in vitro* using the Fenton reaction. Imlay et al. in 1988 found that exposure of *Escherichia coli* to low concentrations of hydrogen peroxide resulted in DNA damage that causes mutagenesis and kills the bacteria. Hydroxyl radicals generated by the Fenton reaction (ferrous sulfate + hydrogen peroxide) cause oxidatively induced breaks in DNA strands to open circular or linear. Cuprum salts can also be used for this reaction. Hydroxyl radicals react with nitrogenous bases of DNA producing base radicals and sugar radicals. The base radicals, in turn, react with the sugar moiety causing breakage of the sugar-phosphate backbone of nucleic acid, resulting in strand break. In particular, the effect of verbascoside to protect plasmid pBR322 DNA against the Fenton reaction was studied (Zhao et al., 2005). The plasmid DNA was damaged by hydroxyl radical generated from the Fenton reaction with H_2O_2 and Fe(II) or Fe(III). Such DNA damage was characterized by the diminution of supercoiled DNA forms or by the increase of relaxed or linear DNA forms after oxidative stress. Kaempherol demonstrated a significant DNA-protective effect, including in a study of the Fenton reaction (Simunkova et al., 2021). Another well-known flavonoid luteolin was studied in the Cu-Fenton reaction (Jomova et al., 2022). A dose-dependent protective effect of luteolin against ROS-induced DNA damage was observed. This effect was more pronounced compared to quercetin and kaempferol. DNA protective effect of quercetin against ROS attack was described (Jomova et al., 2017). It suppressed the formation of ROS due to the decreased catalytic action of copper in the Fenton reaction. It inhibited the formation of ROS due to the reduced catalytic effect of copper in the Fenton reaction by chelating and scavenging ROS with free quercetin.

In our study, we detected the ability of extracts from hairy roots rich in different flavonoids to protect the plasmid DNA against damage caused by hydroxyl (·OH) radicals. The reaction mixture contained pUC19 plasmid DNA, Fenton's reagent followed by the addition of different concentrations of the extract. The reaction mixtures were incubated for 30 min at 37°C. After 30 minutes of incubation, bromophenol blue dye was added. The reaction mixtures were loaded on 0.8% agarose gel and electrophoresis was carried out followed by ethidium bromide staining. The supercoiled, open-circular, and linear forms of pUC19 were visualized and quantified using the LAS-4000 MINI Gel Documentation system. The extracts of hairyroots were found to be able to prevent DNA damage (Fig. 34). In the control reaction (red), without extract addition, open circular or linear DNA forms were detected. At the same time, the addition of the extracts from the hairy roots (green) protected DNA, and this protection has led to the minimization of damaged DNA amount and preservation of the native supercoiled form of DNA.

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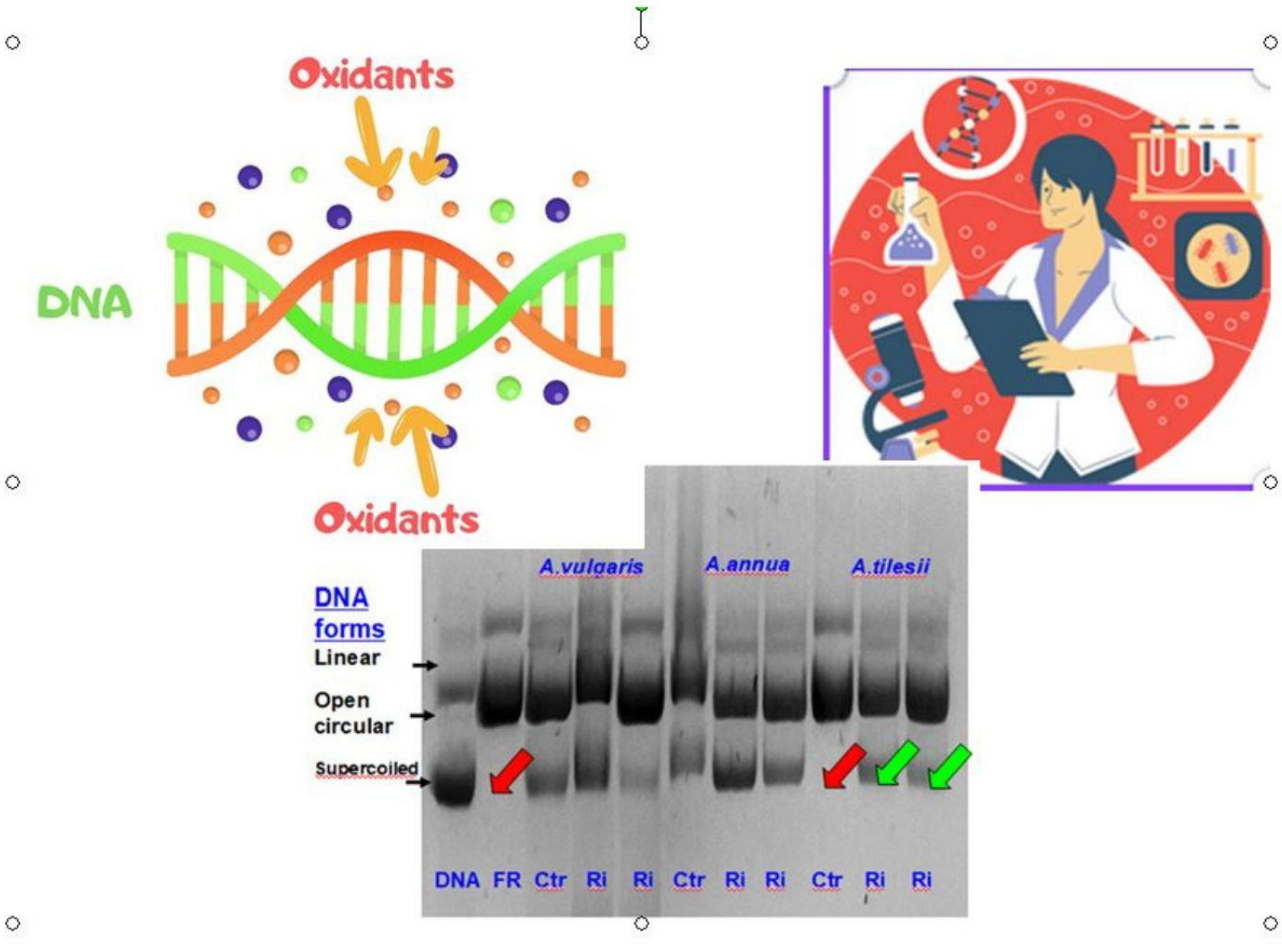

Figure 34 – Hairy root extracts protect DNA from oxidative stress

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3.13. Using of hairy root extracts for nanoparticles initiation

"Green" synthesis refers to the use of natural or plant-based materials in nanoparticle synthesis, and it has become an increasingly popular approach due to its environmental friendliness and low toxicity. NPs can be produced in various sizes and shapes, including spherical, rod-shaped, and triangular (Shankar et al., 2004).

Silver nanoparticles (AgNPs) are one of the most widely studied types of nanoparticles, and they can be synthesized using a variety of plant extracts (Ahmad et al., 2016; Mohanpuria et al., 2008;). One of the advantages of green synthesis is that it allows for a high degree of control over the size and shape of the resulting nanoparticles. AgNPs synthesized using plant extracts tend to be more stable than those synthesized using chemical methods. This is because the plant extracts contain diverse compounds, such as polyphenols and flavonoids that can act as stabilizing agents for the nanoparticles (Vanlalveni et al., 2021). Green-synthesized AgNPs are generally considered to be more biocompatible than those synthesized using chemical methods since they are produced using natural materials. This makes them potentially useful for a range of biomedical applications, such as drug delivery and imaging (Chopra et al., 2022).

AgNPs synthesized using plant extracts have been shown to exhibit potent antimicrobial activity against a variety of bacteria, fungi, and viruses. This is thought to be due to the unique surface properties of the nanoparticles, which can disrupt microbial cell membranes and inhibit their growth (Mohanpuria et al., 2008; Chopra et al., 2022; Abdelghany et al., 2018; Vanlalveni et al., 2011). Overall, the use of plant extracts in the synthesis of silver nanoparticles offers several advantages over traditional chemical methods. However, it's worth noting that the properties of the resulting nanoparticles can vary depending on the type of plant extract used, as well as the specific conditions of the synthesis.

Our study (Kobylinska et al., 2020) was focused on the synthesis of AgNPs using extracts from the hairy roots of *Artemisia tilesii* Ledeb. and *Artemisia annua* L. (Fig. 35). We evaluated the effect of operational parameters such as the type of solvent, the temperature of extraction, flavonoid concentration, and the reducing power of the extracts on the particle size and yield of the resultant nanoparticles. Total flavonoid contents in *A. annua* and *A. tilesii* hairy root extracts were up to 80.0 ± 0.9 and 108 ± 4.4 mg RE per g DW, respectively. UPLC-ESI-UHR-Qq-TOF-MS analysis allowed to identify of some flavonoids luteolin-7-β-D-glucopyranosid, isorhamnetin 3-*O*-glucoside, baicalein-7-*O*-glucuronide, apigenin-7-*O*-glucoside, quercetin, sitosterol, caffeoylquinic, galic, chlorogenic and caffeic acids in the extracts. Due to the presence of these compounds, the extracts demonstrated high reducing activities.

Spherical, oval, and triangular nanoparticles with effective sizes of 5–100 nm were obtained after the addition of the extracts to the $AgNO_3$ solution. The transmission electron microscopy (TEM) data revealed significant differences in the shapes of NPs, obtained from the extracts from different root clones. The clustered and irregular NPs were found in the case of using ethanol extracts, mostly aggregated and having a size of 10–50 nm. The size of AgNPs decreased to 10–30 nm when the aqueous extracts were obtained at 80 °C. Nanoparticles possessed antimicrobial activity, which in some cases was higher than the same activity of 1mM $AgNO_3$ solution.

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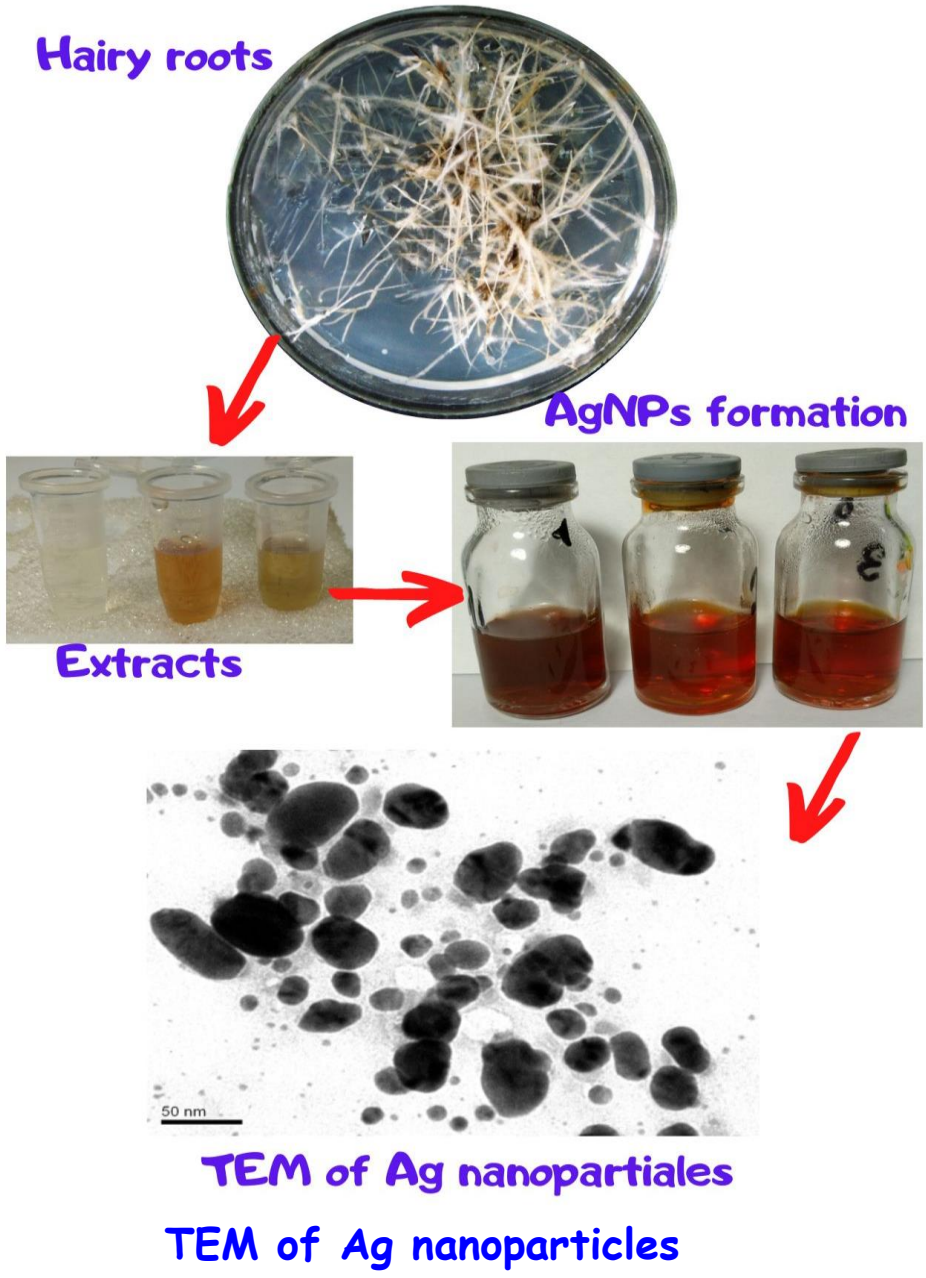

Figure 35 – Hairy root extracts can be used for "green" synthesis of silver nanoparticles

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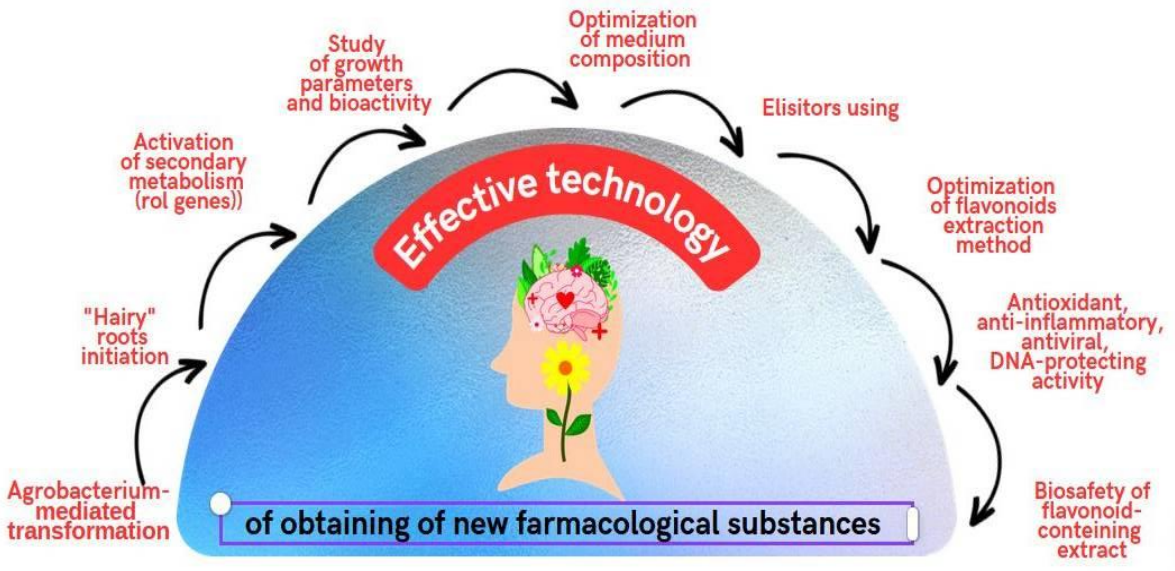
In this chapter, we have shown a wide range of possibilities for using hairy roots. Since genetic transformation can lead to significant changes in the metabolism of plant cells, such roots can synthesize compounds specific to these plants in significantly greater amounts than mother plants.

At the same time, transgenic roots can synthesize compounds that are not characteristic of the original plants. It is also the result of genetic transformation, transfer, and incorporation of agrobacterial genes into the plant genome and their influence on the activity of the plant's genes.

In particular, we showed the possibility of increasing the content of flavonoids, sugars, and artemisinin in transgenic roots. It has also been determined that extracts from hairy roots can have increased antioxidant and reducing activity, as well as the ability to protect DNA from the damaging effect of oxidative stress.

This indicates great prospects for the possible use of hairy roots as valuable producers of biologically active compounds and a source of raw materials for the creation of new drugs with a wide spectrum of activities.



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3.14. Cultivation of hairy roots in bioreactors

Hairy roots are fascinating creatures, products of the mind and work of scientists. They differ from ordinary plant roots in that they can be grown for unlimited time in *in vitro* culture with periodic subcultivation and replacement of the nutrient medium. The composition of this medium is quite simple (salts of potassium, sodium, calcium, magnesium, and iron) with the addition of a carbon source (for example, sucrose). The cultivation of such biotechnological roots does not require the use of additional high-value components.

These roots can synthesize the same compounds as the roots of plants that grow in the soil in the natural environment. In addition, the content of such compounds may exceed their content in the original mother plants. This is the result of transformation and may be due to the specific activity of bacterial *rol* genes transferred to the genome of plants. All these features make it possible to develop technologies for the long-term cultivation of hairyroots in special equipment – bioreactors.

Growing roots in bioreactors allow to preserve of natural plant populations; avoids the negative impact of weather conditions on plant growth; the constant content of bioactive compounds; gets guaranteed pure plant material without contamination with pesticides, herbicides, etc., since the roots are grown on a nutrient medium of a standardized composition; free up additional areas that can be used for crops growing; increase the concentration of bioactive compounds synthesized by plants due to the use of highly productive root lines (Fig. 36).

The main conditions necessary for the growth of roots in a bioreactor are the nutrient medium of the required composition and the oxygen (one or another method of aeration, for example, mixing or bubbling air). The nutrient medium can be in the bioreactor permanently, filling a part of its volume, or as a fog, which allows the roots to have contact with oxygen in the air.

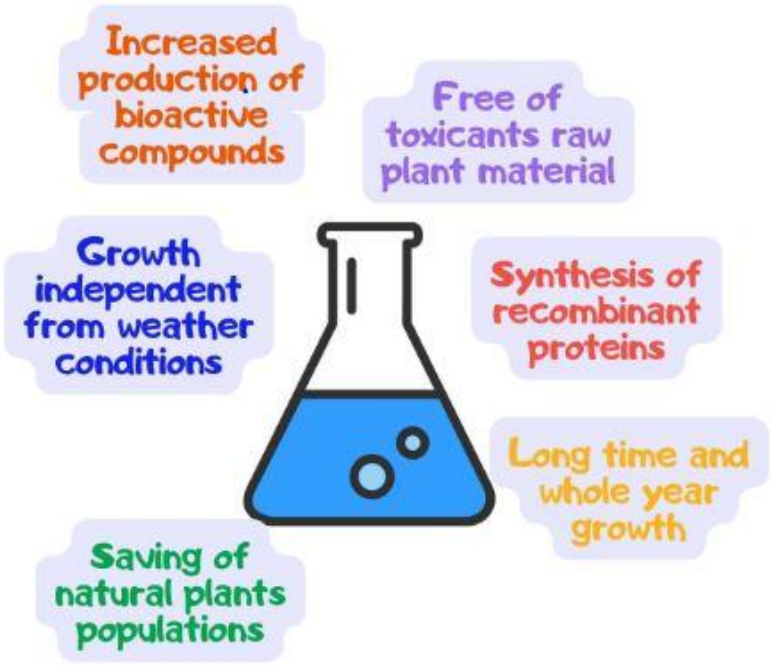

Figure 36 – Advantages of using bioreactors for the cultivation of hairy roots

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Bioreactors were used for the cultivation of different hairy roots to produce recombinant bioactive compounds. Some publications are cited below:

a) *Cucumis melo* L. hairy roots were obtained using binary plasmid p221 that included cauliflower mosaic virus 35S promoter, tobacco etch virus (TEV) leader sequence, and 35S terminator. The roots were cultivated in an 18-Litter bioreactor for the production of human tissue-plasminogen activator (t-PA) protein with a maximum content of t-PA 0.46 μg/mg total soluble protein (Kim et al., 2012b);

b) *Solanum lycopersicum* L. hairy roots synthesized recombinant protein containing a fusion of rabies glycoprotein (RGP) and ricin toxin B (RTB) chain (rgp–rtxB) in 5 L bioreactor with biomass yield of 197.4 g/L and RGP RTB 7.84 μg/g (Singh et al., 2015);

c) *Brassica oleracea* var. Italica hairy roots carried pCAMBIA1105.1 binary vector accumulated isoform 1 of the human growth hormone (hGH1) in case of cultivation in 1.5-Litter mesh airlift bioreactor (López et al., 2014). The content of hGH1 7.8 ± 0.3 μg/g DW was for the bioreactor.

Hairy roots of plants of various species were used for cultivation in bioreactors. References to some of them are provided below (Kintzios et al., 2004; Eungsuwan et al., 2021; Jeong et al., 2003; Suresh et al., 2005; Mehrotra et al., 2007; Sivakumar et al., 2010; Mišić et al., 2013; Kuźma et al., 2009; Srivastava and Srivastava, 2012) (Table 4).

Table 4 – Examples of study of hairy root growth in bioreactors

Plant species	**Product/parameter**	**References**
Arachis hypogaea	trans-resveratrol, trans-arachidin-1, trans-arachidin-3	Eungsuwan et al., 2021
Panax ginseng	growth parameters	Jeong et al., 2003
Pueraria phaseoloides	puerarin	Kintzios et al., 2004
Tagetes patula	growth parameters	Suresh et al., 2005
Glycyrrhiza glabra	growth parameters	Mehrotra et al., 2007
Artemisia annua	growth parameters	Sivakumar et al., 2010
Arachis hypogaea	growth parameters	Sivakumar et al., 2010
Centaurium maritimum	secoiridoid glycosides	Mišić et al., 2013
Salvia sclarea	diterpenoids	Kuźma et al., 2009
Azadirachta indica	azadirachtin	Srivastava and Srivastava, 2012
Picrorhiza kurroa	picroliv	Verma et al., 2015
Beta vulgaris	betalaine	Suresh et al., 2004
Hyoscyamus niger	salicylic acid	Kareem et al., 2019

Since the cultivation of hairy roots is of considerable commercial interest, reactors of various types have been developed for about forty years. They differ in technical complexity and design features. However, when designing bioreactors of all types, the need to supply and change the nutrient medium, aeration, and temperature control is taken into account. Such types of bioreactors can be named as Liquid-Phase, Gas Phase, Stirred Tank, Bubble Column, Radial Flow, Nutrient Mist, Trickle Bed, Convective Flow, Rotating Drum, Turbine Blade, and Airlift Bioreactors (Stiles and Liu, 2013; Baqueet al., 2012; Georgiev et al., 2013; Kowalczyk et al., 2022; Srivastava and Srivastava, 2007; Mishra and Ranjan, 2008; Ramakrishnan et al., 2004; Martin and Vermette, 2005; Cuello and Yue, 2008). The construction schemes of some types of bioreactors are presented in the publications of Kim *et al.*, 2002b; Kondo *et al.*, 1989; Kim *et al.*, 2003; Weathers et al., 1997; Srivastava and Srivastava, 2007 and others.

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3.15. The collection of hairy roots: initiation and study

The Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine currently has an extensive collection of hairy roots of medicinal plants of various species, which includes about a hundred collection specimens. We used *Agrobacterium rhizogenes* – mediated transformation to obtain clones of hairy roots of different medicinal plants of *Artemisia vulgaris, A. annua, A. dracunculus, A. absinthium, A. tilesii, Bidens pilosa, Cichorium intybus, Althaea officinalis, Tragopogon porrifolius* and other plant species (Fig. 37).

We studied the possibility of using the genetic transformation of different medicinal plants to obtain hairyroot cultures. These roots were studied as producers of the complex of bioactive compounds with a broad spectrum of bioactivity. The clones differed in some physiological parameters (growth rate) and the content of secondary metabolites. The clones from the collection can be used for the production of a complex of bioactive compounds (polyphenols, flavonoids, artemisinin) with a high level of antioxidant, anti-inflammatory, and antiviral activity.

Artemisia annua **L.** (**sweet wormwood, annual wormwood**) is the most studied and best-known plant of this genus. These plants are native to Asia but have become widespread in different parts of the world. *A. annua* became especially famous after the awarding of the 2015 Nobel Prize to the Chinese researcher Tu Youyou for her long-term study of annual wormwood as a producer of artemisinin, a compound with antimalarial properties. The plants synthesize coumarins, flavones, flavonols, phenolic acids, sesquiterpenes, monoterpenes, and other bioactive compounds (Septembre-Malaterre et al., 2020; Sanjay Kumar Rai et al., 2021). *A. annua* plants can be used not only for malaria treatment (Willcox, 2009; Hsu, 2006; Ho et al., 2014; Sankhuan et al., 2022). They possess hepatoprotective, anti-inflammatory, anti-cancer, antioxidant, anti-HIV and antimicrobial properties (Alesaeidi et al., 2016; Ćavar et al., 2012; Lubbe et al., 2012; Kim et al., 2014; Slezakova et al., 2017; El-Askary et al., 2019; Lang et al., 2019; Li et al., 2015; Ekiert et al., 2021).

Annual wormwood plants were used for the construction of transgenic plants and the initiation of hairy root growth. The ideas of these works were based on the necessity to develop the transformation protocol for obtaining plant or root clones with increased levels of bioactive compound accumulation. For this purpose, different strains of *A. tumefaciens* and *A. rhizogenes* were used. In particular, Vergauwe et al. (1996) compared different *A. tumefaciens* strains were used for A. annua genetic transformation and obtained regenerated plants on the medium with 0.05 mg/L naphthaleneacetic acid and 0.5 mg/L N(6)-benzyladenine. This work was one of the first attempts at transgenic wormwood plant construction.

Different biotechnological approaches increased artemisinin and endoperoxide sesquiterpene lactone content (Tang et al., 2014). This plant-produced compound is of great interest because of its use as an effective antimalarial drug. For instance, artemisinin biosynthesis enhancement was studied by downregulation of the β-caryophyllene synthase gene (Chen et al., 2011). Fu et al. (2021) detected that overexpression of blue light receptor AaCRY1 increased artemisinin content in transgenic plants. Heterologous expression of cyanobacterial PCS increased artemisinin content in *A. annua* hairy roots (Pandey et al., 2021b). LIS promoter activity for artemisinin synthesis was analyzed (Wang et al., 2014). Overexpression of artemisinic aldehyde Δ11 (13) reductase gene enhanced artemisinin and its relative metabolite biosynthesis in transgenic plants (Yuan et al., 2015). It was studied that *A. annua* plants overexpressing a pleiotropic drug resistance transporter gene *AaABCG40* showed higher artemisinin content (Fu et al., 2020). Transgenic plants that

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carried the *rol* B gene showed a 1.2-12-fold increase in artemisinin, artesunate, and dihydroartemisinin content (Dilshad et al., 2015a). Transfer of the *rol* C gene also increased to artemisinin, artesunate, and dihydroartemisinin concentration. However, the level of synthesis under such conditions was lower than in the case of *rol* B gene use. Expression of β-glucosidase (Singh et al., 2016), HMG-CoA reductase gene (Aquil et al., 2009), cyanobacterial PCS (Pandey et al., 2021b), and AaWRKY1 (Han et al., 2014) affected artemisinin biosynthesis in transgenic plants and hairyroots.

A. annua hairy roots were induced by the leaf treatment with the LBA 9402 strain of *A. rhizogenes.* These root cultures produced the sesquiterpenes artemisinic acid and arteannuin B. It should be noted that hairy roots were observed to regenerate spontaneously into plantlets on a hormone-free MS medium (Banerjee et al., 1997). Liu et al. (1998) obtained A. annua hairy roots and studied the dynamics of growth and biosynthesis of artemisinin. The dynamics of root growth in different culture systems were evaluated by Kim et al. (2003). Wang et al. (2009) studied hairy root culture and the effect of different compounds (cerebroside and nitric oxide) as elicitors for increasing artemisinin production. Weathers et al. (2004) obtained *A. annua* hairy roots and studied the effect of sugars on growth.

In our laboratory wormwood hairy roots were initiated after the transformation by leaves cocultivation with *A. rhizogenes* A4 wild strain and, in addition, bacteria carried plasmid with human interferon α2b gene were used (Drobot et al., 2016a; Matvieieva, 2015) (Fig. 38).

Artemisia absinthium **L. (wormwood, grand wormwood, absinthe**) (Fig. 39) is a perennial medicine plant. It synthesizes phytoncides and demonstrates insecticidal properties. The plants are used in anorexia and indigestion treatment. The aerial parts of the plants have anthelmintic, anti-cancer activity (Blagojević et al., 2006; Julio et al., 2017; Sultan et al., 2020; Mohammed, 2022). They accumulate antioxidants, can alleviate liver inflammation, demonstrate a reduction in cholesterol levels, etc (Batiha et al., 2020; Anibogwu et al., 2021; Lachenmeier, 2010). The plants are known as producers of thujone (Bach et al., 2016). *A.absinthium* extract is known to have antioxidant (Bora et al., 2011; Singh et al., 2011), immunomodulatory (*Shahnazi* et al., 2015), wound-healing (Boudjelal et al., 2020), anti-inflammatory, analgesic (Amrollahi et al., 2014), antitumor (Koyuncu, 2018), antiulcer (Shafi et al., 2004), antibacterial, antifungal (Kordali et al., 2005; Obistioiu et al., 2014) activities, as well as neuroprotective (Bora and Sharma, 2011), hepatoprotective (Amat et al., 2010), hypoglycemic (Daradka et al., 2014) effects. Such a wide range of properties is related to the *A. absinthium* chemical composition. Such compounds as lactones, terpenoids, essential oils, organic acids, resins, tannins, and phenols were previously detected in the extracts of this plant (Batiha et al., 2020). For example, isolated dimeric guaianolides found in *A. absinthium* exhibited cytotoxic activity, inhibited cyclooxygenase-2, and had anti-HIV-1 protease activity (Turak A. et al., 2014).

Nin et al. (1997) demonstrated the possibility of the genetic transformation of *A. absinthium*. Hairy roots were produced after cocultivation of the shoots with *A. rhizogenes* strains 1855 and LBA 9402. The obtained roots were characterized by a high growth rate and a 463-fold increase in dry weight after 28 days of cultivation in the medium supplemented with 40 g L^{-1} of sucrose. Using gas chromatography/mass spectrometry analysis the authors analyzed secondary metabolite content in the hairyroots – a mixture of 50 compounds.

In our laboratory *A. absinthium* hairy roots were obtained using *A. rhizogenes* with pCB124 plasmid (Olkhovska et al., 2021). Hairy roots (Fig. 39) were characterized by a higher phenolic content, particularly flavonoids (up to 4.784 ± 0.10 mg/g FW) compared to the control (3.861 ± 0.13 mg/g FW). Also, the extracts from transgenic roots demonstrated

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higher antioxidant activity in the reaction with the DPPH reagent (EC_{50} = 3.657 mg) compared with extracts from the control plants (EC_{50} = 6,716 mg).

Figure 37 – The hairy roots from the collection (Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine)

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Figure 38 – *Artemisia annua* hairy roots (Matvieieva et al., 2016)

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Figure 39 – Initiation of *Artemisia absinthium* hairy roots using *A. rhizogenes* and differences in growth rate (Olkhovska et al., 2021)

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***Artemisia tilesii* Ledeb.** known as **Aleutian mugwort** (Fig. 40) is a perennial herb of the Compositae family. These plants naturally grow in Japan, northern North America, and Alaska. It possesses resistance to different stress factors, for example, a range of pH and low temperature (Adams et al., 1984). These plants also are characterized by antirheumatic, disinfectant, deodorant, and anti-tumor effects. They are used in Alaska's traditional medicine to treat fever, infection, tumors, arthritis and other joint pains, bleeding, congestion, and tuberculosis (Overfield et al., 1980; Griffin, 2001). Unfortunately, studies of these fascinating plants are practically not carried out. However, our laboratory has been studying these plants as an object for biotechnological experiments for more than ten years. In particular, we have created a collection of hairy roots of plants of this species and are conducting comprehensive studies of the bioactivity of the extracts (Matvieieva et al., 2016; Matvieieva et al., 2020).

We were the first to obtain Aleutian mugwort hairy roots (Matvieieva et al., 2016) by an efficient and rapid protocol. Leaf explants were cocultivated with *A. rhizogenes* A4 wild strain or *A. rhizogenes* carrying the plasmids with *nptII* and *ifn-α2b* genes. Root formation on the explants started in 5–6 days after their cocultivation with bacterial suspension. These root cultures were used in different experiments to study of peculiarities of their growth (growth rate), accumulation of bioactive compounds (Duplij, Matvieieva, 2019; Matvieieva et al., 2020), resistance to stress factors (Matvieieva et al., 2019b) and possibility of nanoparticles synthesis (Kobylinska et al., 2022).

***Lactuca sativa* L. (lettuce)** is the most often grown leaf vegetable. It is low-calorie and low-fat, but rich in fiber and essential minerals (Kim et al., 2016). Lettuce is also a source of various phytochemicals. Such compounds as glycosylated flavonoids, phenolic acids, the vitamins A, B, and K, ascorbic acid, tocopherols, and sesquiterpene lactones lactucin and lactucopicrin were identified in the plants (Assefa et al., 2019; Bahorun et al., 2004; Becker et al., 2015; Bunning et al., 2009; Chun et al., Damon et al., 2005; Liu et al., 2007; Yang et al., 2018). These components are nutritional bioactive compounds. Lettuce plants possess different health-beneficial properties due to the synthesis of these secondary metabolites. For example, lettuce demonstrated anti-inflammatory, anti-diabetic, anti-depressant, anti-coagulant, anticancer, antimalarial, and radical scavenging activity (Adesso et al., 2016; Gan et al., 2016; Bischoff et al., 2004; Blasco et al., 2008; Cheng et al., 2014; Gopal et al., 2017; Ismail et al., 2015; Yang et al., 2022).

Ismail et al. (2019a, b) studied the effect of the genetic transformation and bacterial rol gene transfer on the secondary metabolism of lettuce plants. They found the differences between untransformed lettuce and the plants transformed with *rolABC* or *rolC* genes. For instance, ferulic acid levels increased 3033-9777%, aminooxononanoic acid increased 1141-1803%, and 2,3,5,4'tetrahydroxystilbene-2-O-β-d-glucoside increased 40,272-48,008%. The plants transformed by *rol*ABC genes (Ismail et al., 2017) also significantly differed from the mother plants in their bioactivity. In particular, the transformed plants showed 91-102 % and 53-65 % increase in total phenolic and flavonoid contents compared to untransformed plants. The total antioxidant and reducing activity also increased in the transformed plants. The authors studied the enhanced antidepressant and anticoagulant potential of these plants compared to the control.

We obtained lettuce hairy roots after the transformation by *Agrobacterium rhizogenes* with the human interferon α2b gene (Fig. 41) and studied the possibility of plant regeneration from these roots (Matveeva et al., 2012a). The regenerated plants differed from the plants of wild type by elongated internodes, early flower-bearing stem formation, and purple coloration of leaves. The transgenic root extracts demonstrated antiviral activity of 1620...5400 IU (Matveeva et al., 2012b).

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Figure 40 – *Artemisia tilesii* hairy roots (Matvieieva et al., 2016)

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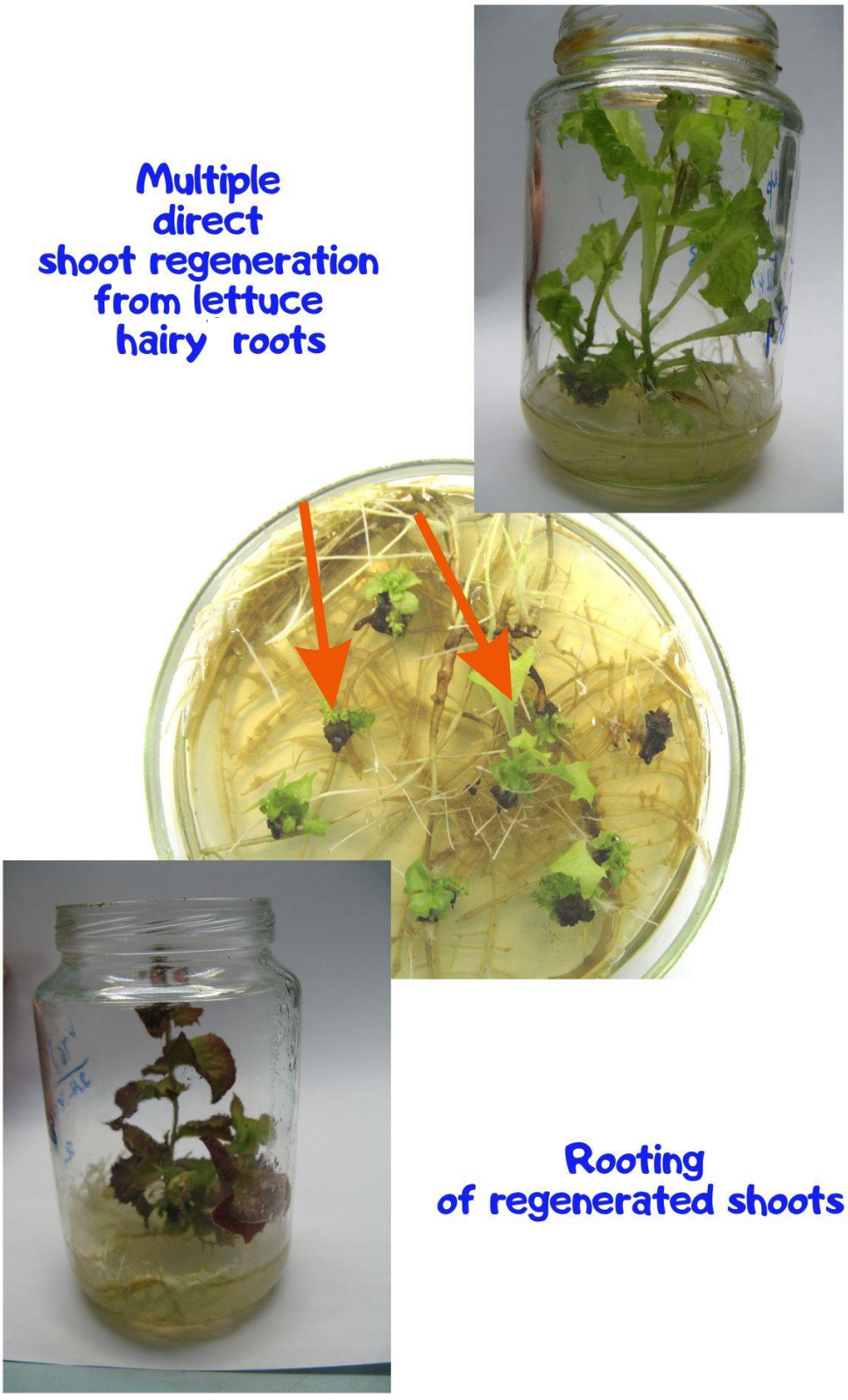

Figure 41 – *Lactuca sativa* hairy roots and regenerated plants

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***Artemisia vulgaris* L.**, or **common mugwort** plants are native to different regions (Europe, Asia, and North Africa) and naturalized in North America. The plants are used as raw material due to the synthesis of different bioactive compounds: essential oil, flavonoids, and sesquiterpenoids lactones, including artemisinin (Lee et al., 1998; Tigno et al., 2000; Blagojević et al. 2006; Judžentien et al. 2006; Natividad et al., 2011; Abad et al., 2012; Abiri et al., 2018; Madhav et al., 2018; Numonov et al., 2019; Nganthoi et al., 2019; Malik et al., 2019).

It is listed in the European Pharmacopoeia and is used in traditional Chinese, Hindu, and European medicine. The plant extracts are known for their antioxidant, hepatoprotective, antihyperlipidemic, antimalarial, anti-inflammatory, antispasmolytic, antinociceptive, estrogenic, cytotoxic, antispasmodic and bronchodilator, antibacterial, larvicidal, and antifungal effects (Lee et al., 1998; Gilani et al., 2005; Temraz et al., 2008; Pires et al., 2009; Khan et al., 2009; Erel et al., 2011; Kodippili et al., 2011; Hiremath et al., 2011; Raj Singh et al., 2011; Govindaraj et al., 2013; Afsar et al., 2013; Obistioiu et al., 2014; Saleh et al., 2014; Khan, 2015; El-Tantawy et al., 2015; Oyedemi et al., 2015; Ekiert et al., 2020; Ben Nasr et al., 2020; Pandey et al., 2021). The plants were studied as a potential source of antioxidant phenolic compounds (Melguizo-Melguizo et al., 2020). European French Pharmacopoeias listed these plants as a possible homeopathic raw material.

Various aspects of the use of *A. vulgaris* plants in biotechnological research have been identified. In particular, the conditions under which the regeneration of shoots of these plants *in vitro* was studied (Borzabad et al., 2020).

A methodology for the genetic transformation of wormwood plants has been developed. For example, four *A. rhizogenes* strains were used for the genetic transformation of *A. vulgaris* (Sujatha et al., 2013). The authors studied the potential of different kinds of explants (hoot tip, leaf, and node) for hairyroots obtaining. The A_4GUS strain was more competent for this purpose. Its application for leaf explant transformation allowed initiating hairy roots with the highest transformation rate (92.6%). Four media compositions based on the ½MS medium were compared for biomass production. Effect of growth conditions (the composition of the culture medium, addition of Farnesyl Diphosphate precursor and vitamins) on initiated hairy roots growth index was evaluated (Balasubramani et al., 2021).

In our laboratory, the transformation was carried out by a wild strain of *Agrobacterium rhizogenes* A4 and agrobacteria carrying the human interferon-a2b (ifn-a2b) gene. Lines of transgenic roots of *A. vulgaris* differed significantly in the content of biologically active compounds: artemisinin (0.237–1.020 and 0.687 mg/g of dry weight, respectively, in transgenic lines and control) and fructans (32–136 and 264 mg/g of dry weight, respectively, in transgenic lines and controls).

These data confirmed that the method of *A. rhizogenes*-mediated transformation can produce transgenic roots of *A. vulgaris* with an increased content of artemisinin, a compound with antimalarial properties. (Drobot et al., 2017; Matvieieva et al., 2019a) (Fig. 42).

The content of artemisinin and fructans in the transgenic roots of *A. vulgaris*, SOD, and the antioxidant activity (AOA) of extracts of these roots were determined (Drobot et al., 2015; Drobot et al., 2016b, in Ukrainian).

The roots from the collection were used for metal nanoparticles obtaining. The ethanol extracts, reach in flavonoids, addition to the $AgNO_3$ solution resulted in silver nanoparticles (AgNPs) synthesis (Kobylinska et al., 2020). The extracts were also used for magnetic Fe nanoparticles obtaining (Kobylinska et al., 2022).

The peculiarities of the synthesized silver nanoparticles, as well as magnetic nanoparticles (physical characteristics, bioactivity), were studied (Kobylinska et al., 2020; Kobylinska et al., 2022).

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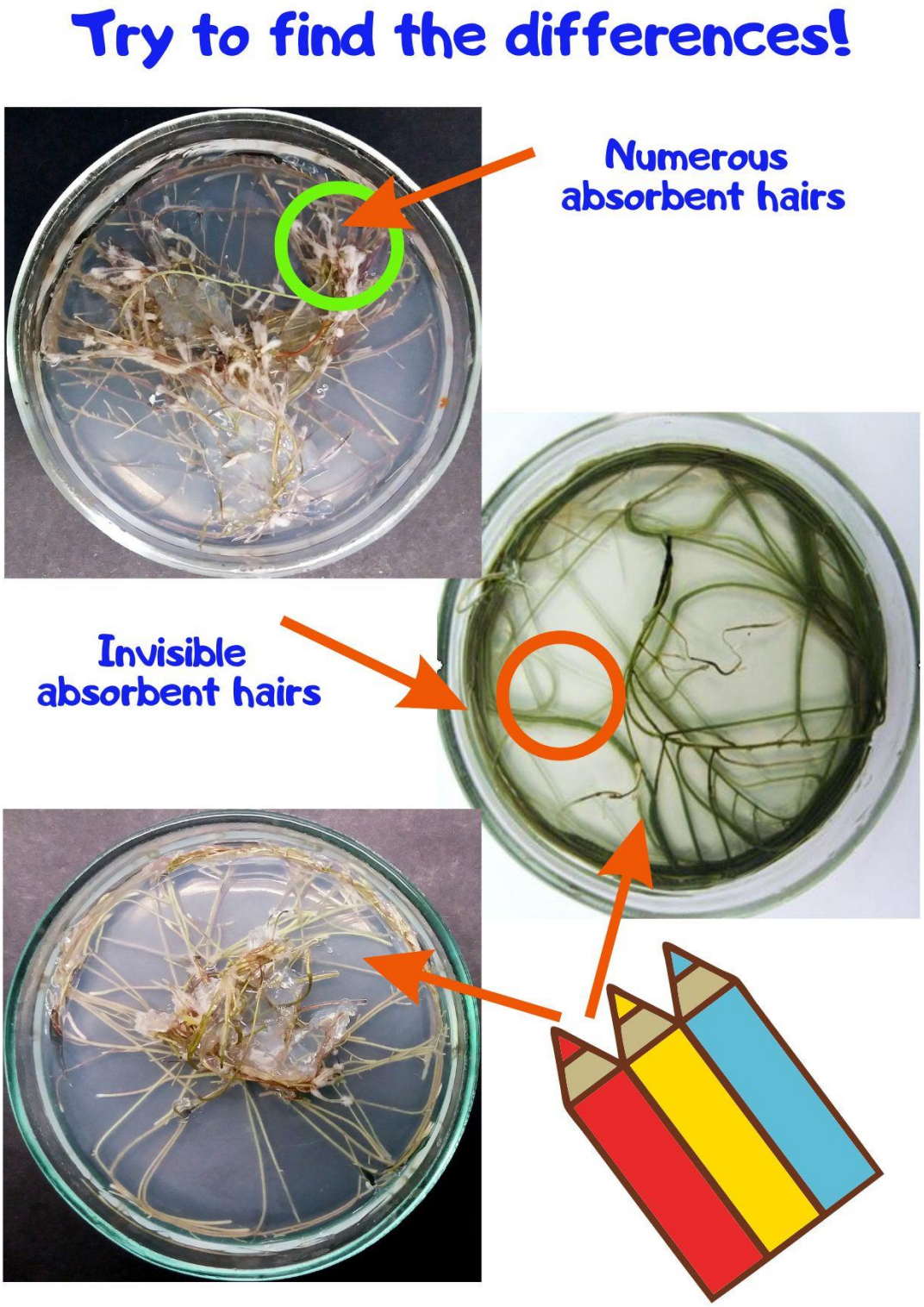

Figure 42 – *Artemisia vulgaris* hairy roots

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***Artemisia ludoviciana* L., known as silver wormwood, or Louisiana wormwood**, is a rhizomatous perennial plant, native to North America. It is cultivated as an ornamental plant. This species has been medicinally used in Mexico since pre-Columbian times, and is popularly known by names such as "estafiate", "istafiate" "ambfe", "ajenjo" and "artemisia", among others. The plants are used in traditional medicine for the treatment of inflammation, bronchitis, and digestive ailments such as gastritis (Esquivel-García et al., 2018; Nicholson et al., 1991; Mata et al., 2019; Alonso-Castro et al., 2017). Aboriginal Australians used them to treat dermatology problems (*Hellson, 1974*). *A. ludoviciana* extracts were tested as a drug against gastritis initiated by *Helicobacter pylori* (Palacios-Espinosa et al., 2021). Gastroprotection, as well as anti-inflammatory effects, were studied. Estafiatin and eupatilin were isolated and exhibited anti-*H. pylori*. The plants demonstrated antimicrobial and antioxidant activity (Lopes-Lutz et al., 2008; Jimenez-Arellanes et al., 2003). It is also active against *Vibrio cholerae* (Garcia et al., 2006) and possesses antiprotozoal activity (Fernández et al., 2005).

Presently, there are only a few publications on the study of the biological characteristics of plants of this species, and there are no publications on genetic transformation. That is why the recent article of *Sánchez-Ramos* et al. (2022) attracts attention. The authors studied achillin production in *A. ludoviciana* in different conditions (photoperiod and darkness conditions). They used MS medium with 0.1 mg/L of kinetin or benzyl amino purine and/or naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid and 4-amino-3,5,6-trichloro-2-pyridine carboxylic acid at 0.1 and 1.0 mg/L for callus induction. GC-MS analysis showed higher achillin content (1703.05 μg/mL) in leaf calluses with PIC (1.0) and KIN (0.1) under photoperiod, and in node plantlets (1880.01 μg/mL) with PIC (0.1) and BAP (0.1). For the first time, we performed the genetic transformation of plants of this species using *A. rhizogenes* and obtained hairy roots with the human interferon-α2b gene (unpublished results, Fig. 43).

***Artemisia dracunculus* L. (tarragon)** is widespread throughout the world perennial plant and also grows in Ukraine (Boiko, 2013). The use of *A. dracunculus* was mentioned in ancient Greece. Plants are known to synthesize high amounts of essential oil. So tarragon is used in folk medicine, cosmetics, and cuisine.

Flavonoids, coumarins, phenylpropanoids, and terpenes determine the antimicrobial, antiviral, antifungal, and antioxidant activities of *A. dracunculus*. Such a broad spectrum of biological activities could cause tarragon`s use in the pharmaceutical industry for the treatment of diseases such as inflammation, hepatitis, and different kind of infections (bacterial, viral) (Eidi et al., 2016; Mohsenzadeh, 2007; O'Mahony et al., 2005; Obistioiu et al., 2014). Leaves of *A. dracunculus* accumulate artemisinin up to 0.27% (Mannan et al., 2010). Studies of *A. dracunculus* were devoted to plant micropropagation, and medicine compound accumulation (Fernández-Lizarazo et al., 2012; Obolskiy et al., 2011).

In our laboratory, we studied (Drobot et al., 2016c) the possibility of *A. dracunculus* genetic transformation (Fig. 44). We obtained the transgenic hairy root cultures using *A. rhizogenes* A4 – mediated transformation. It was shown that leaves of *in vitro* cultivated plants were the optimal type of explants. The transgenic root formation frequency was up to 20% in the case of leaf usage. The time of explants and bacteria cocultivation had a crucial effect on the frequency of transgenic root formation. The optimal time of explant cultivation on the medium without cefotaxim for agrobacterial gene transfer into plant cells appeared to be four days. Prolongation of this term has led to explants' death while reducing was not successful for root obtaining. Roots were formed on the 7th day after cocultivation with bacterial suspension on leaf explants. Transgenic root lines differed in morphological features and growth rate.

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Figure 43 – *Artemisia ludoviciana* hairy roots

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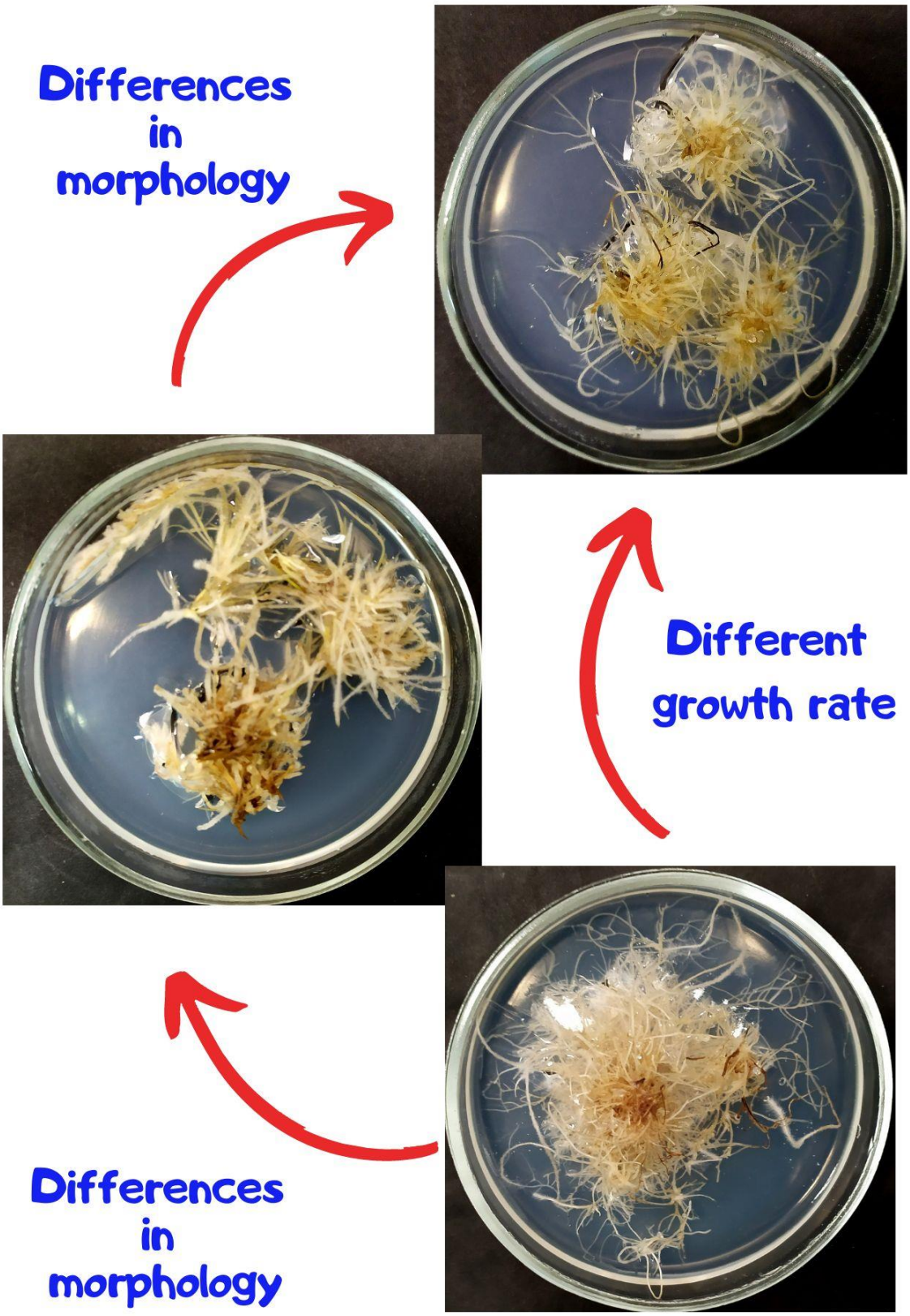

Figure 44 – *Artemisia dracunculus* hairy roots

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***Althaea officinalis* L., or marshmallow**, is a perennial medicinal plant from the Malvaceae family. The plant originates in India but is now widespread in Europe, America, Asia, and North Africa. *A. officinalis* has long been used as a medicinal plant because it has antiseptic, antioxidant, antimicrobial, anti-inflammatory, and gastroprotective properties. *A. officinalis* preparations (powder, aqueous infusion, liquid extract, syrup) are used for diarrhea, acute gastritis, enterocolitis, and as an expectorant for catarrhal conditions of the respiratory tract. It is also used for asthma treatment. Althea roots contain up to 35% of mucous substances, which determine the healing properties of the plant, as well as starch (up to 37%), sucrose (10.2%), betaine (up to 4%), and fatty oil (up to 1.7%), astragalin, mucopolysaccharides, arabinofuranan, caffeic acid, chicory, coumarin and coumarin acid, diosmetin, kaempferol, luteolin, quercetin, scopolin (Shah et al., 2011).

Sadighara et al. (2012) studied the dependence of antioxidant activity and flavonoid content on flower color. Flavonoid content was highest in white flowers. Root extracts of *A. officinalis* protected human macrophages against H_2O_2-induced cytotoxicity and H_2O_2-induced ROS production (Bonaterra et al., 2020). Thus, they demonstrated antioxidant and anti-inflammatory activity. Xue et al. (2022) found that *A. officinalis* extracts are potent antioxidants and possess high *α*-glucosidase, 5-lipoxygenase, and nitric oxide inhibitory activities. Rat treatment with the plant extract significantly increased serum HDL cholesterol levels without effects on stool cholesterol and triacylglycerol (Hage-Sleiman et al., 2011). A general decrease in liver enzyme activities and an inhibition of inflammation were observed in this study.

hairyroots of *A. officinalis* were produced using *A. rhizogenes* (Drake et al., 2013). The authors obtained wild-type lines and the roots expressing the cyanovirin-N (CV-N). Different *A. rhizogenes* strains (A4, A13, ATCC15834, and ATCC $15834_{(GUS)}$) were used for marshmallow hairyroot induction (Tavassoli & Safipour Afshar, 2018). In our study (Matvieieva et al., 2013) hairyroots were obtained using *A. rhizogenes* A4 carried pCB161 plasmid with human interferon-α2b gene (Fig. 45). The clones of transgenic roots differed in mass increment from 0, 036 ± 0,008 up to 0,371 ± 0,019 g in 30 days of cultivation and fructan synthesis (from 67,2± 4,47 up to 154,6 ± 6,62 mg/g roots dry weight). Extracts from hairyroots were characterized by high antiviral activity against vesicular stomatitis virus – up to 26 000 IU/ g of roots fresh weight.

***Bidens pilosa* L. (beggartick, Spanish needles, devil needles, black jack)** is an annual plant native to the South American region and distributed in most pantropical areas of the world. Native Amazonians, Australians, and Hawaiians used the plants as edible ones and herbal tea. In Africa, plants are used in folk medicine for human health (Orech et al., 2007). Different constituents (polyacetylenes, polyacetylene glycosides, flavonoids, flavone glycosides, essential oils, chalcones, okanin glycosides, phenolic acids, terpenes, fatty acids, and phytosterols) were identified in *B. pilosa* plants (Xuan et al., 2016; Silva et al., 2011; Chang et al., 2000; Khanh et al., 2009; Priestap, Bennett, 2008; Zhao et al., 2004; Lee et al., 2008). Due to the synthesis of these compounds, *B. pilosa* plants have anti-inflammatory, anti-diabetic, antihyperglycemic, chemopreventive, antimalarial, and antibacterial activity (Yan et al., 2022; Ubillas et al., 2000; Chiang et al., 2007; Chang et al., 2004; Xin et al., 2021; Chien et al., 2009; Tobinaga et al., 2009; Chiang et al., 2004; Oliveira et al., 2004; Deba et al., 2008). The leaf extract is also used to cure malaria, stomach and mouth ulcers, and diarrhea (Subhuti, 2013). The herbicidal and fungicidal activities of the plants were studied (Deba et al., 2007).

B. pilosa hairy roots (Fig. 46) were obtained firstly using *A. rhizogenes* A4 carried pCB124 plasmid with human interferon-α2b gene (Matvieieva et al., 2015a). The extracts possessed antiviral activity 1620...5400 IU/g weight.

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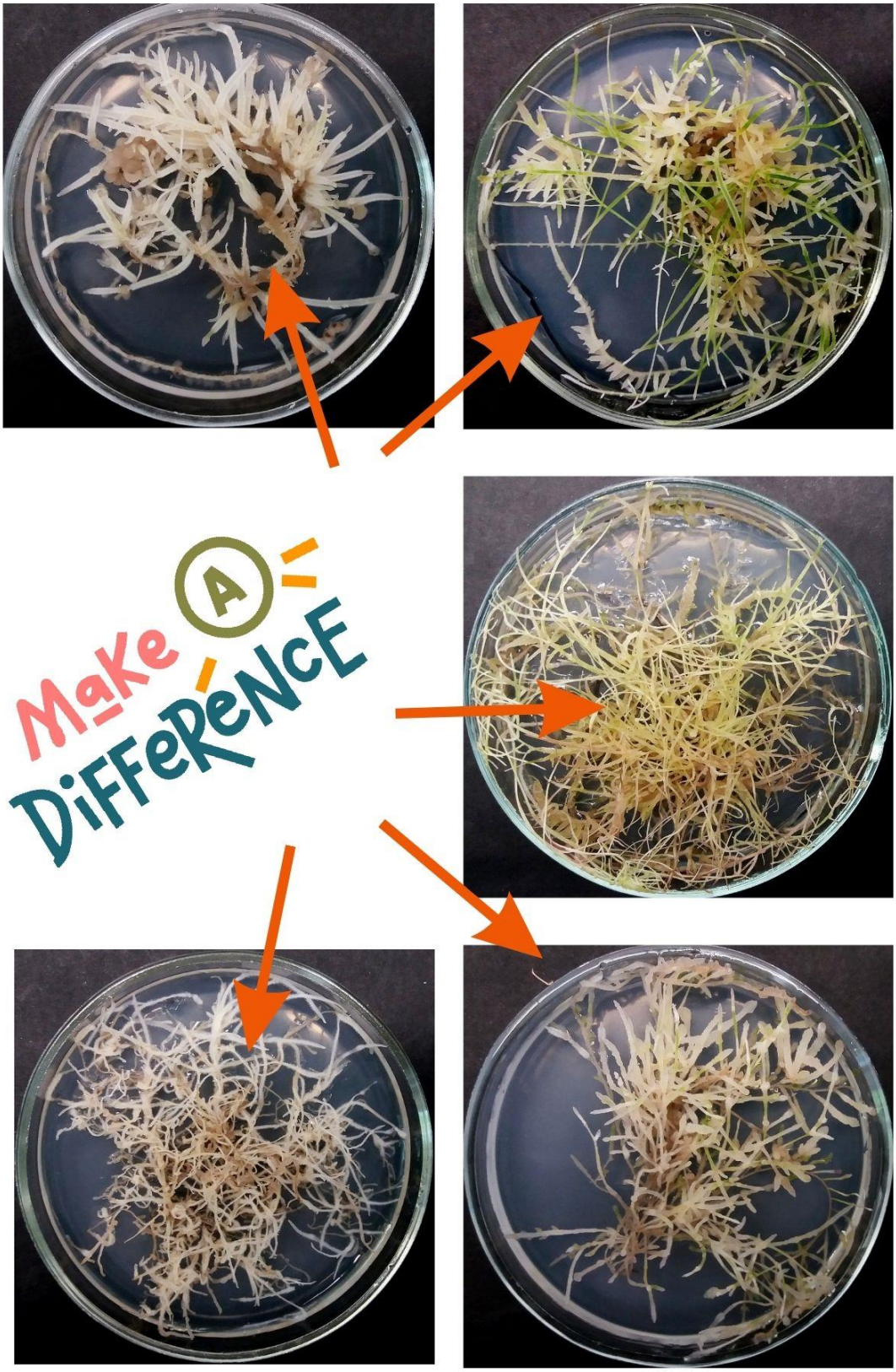

Figure 45 – Morphological differences in *Althaea officinalis* hairy roots (Matvieieva et al., 2013)

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Figure 46 – *Bidens pilosa* hairy roots

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***Cichorium intybus* L., or common chicory,** is a perennial herb from the *Cichorium* genus, *Asteraceae* family. The plants are cultivated worldwide. Chicory is used in the food industry as a salad, for teas and tea blends, for coffee supplementation, and as a source for inulin production (Twargowska et al., 2022). Polyphenols, inulin, oligofructose, and sesquiterpene lactones are present in chicory plants (Dalar et al., 2014; Perovic et al., 2021).

This plant exhibits anti-inflammatory, immunostimulatory, antioxidant, antiulcer, antitumor, and cardiotonic activity and is used to treat diabetes and other diseases (Ki et al., 1999; Gadgoli et al., 1997; Ahmad et al., 1998; Hughes et al., 2001; Monde et al., 1990; Azay-Milhau et al., 2013).

Chicory has a high ability for plant regeneration from various explants. This process has been studied for more than 50 years. For example, leaves (Rehman et al., 2003; Mei et al., 2004; Yucesan et al., 2007; Velayutham et al., 2007; Profumo et al., 1985) and roots (Velayutham et al., 2007; Profumo et al., 1985) can be used as explant material for the production of chicory plants.

We have previously demonstrated that the regeneration frequency of plants (cv. Palla rossa) from cotyledons was 100% (Matvieieva et al., 2009). Plant regeneration is possible through callus formation (Velayutham et al., 2007; Caffaro et al., 1982), embryos (Sidikou-Seynie et al., 1992; Heirwegh et al., 1985), as well as direct regeneration (Matvieieva et al., 2009; Velayutham et al., 2003). To regenerate plants in *in vitro* culture, the following growth regulators are used: Kinetin (Profumo et al., 1985), α-Naphthalenacetic Acid (NAA) and 2-Isopentenyl Adenine (Sidikou-Seynie et al., 1992), Kinetin and Indole-3-Acetic Acid, Kinetin, and NAA, 6-Benzylaminopurine and IAA, BA and NAA, Thidiazuron and IAA, etc. (Rehman et al., 2003; Yucesan et al., 2007; Nenz et al., 2000).

High-efficient transgenic hairy root induction in chicory was demonstrated (Kabirnataj et al., 2016). Conditions of hairyroot initiation were optimized (Fathi et al., 2019). Malarz et al. (2013) constructed chicory hairyroots and studied them as a source of hydroxycinnamates and 8-deoxylactucin glucoside. The content of these compounds in the root biomass reached 1.5 - 5.5 % of dry weight. Bernard et al. (2020) evaluated the accumulation of 3,5-diCQA in hairyroots potent antioxidant and antibacterial compound. In 2023 was published an article that studied chicory hairy roots produced sesquiterpene lactones and polyphenols as a platform for compound synthesis with antimicrobial activity (Häkkinen et al., 2023).

We obtained chicory hairy root cultures (Fig. 47). In our study, transgenic plants carrying either *ifn*-α2b gene encoding human interferon or *esxA*::fbpB$^{\Delta TMD}$ genes encoding *Mycobacterium tuberculosis* antigens ESAT6 and Ag85B were regenerated from the hairy roots (Matvieieva et al., 2011; Matvieieva et al., 2015b).

The direct shoot regeneration from transgenic roots without a callus formation phase was observed on a regulator-free nutrient medium. Direct shoot regeneration without callus formation was observed in one month of transgenic root cultivation on a selective medium. The shoots were characterized by normal for chicory plants phenotype, rooted on 1/2MS medium and formed flowers *in vitro* and in the soil. These plants regenerated from hairyroots had a transferred bacterial *rol* B gene, as well as a human α2b-interferon gene and a selective *nptII* gene. The transgenes transfer and transcription in the hairyroots and plants were confirmed by the results of RT-PCR and PCR analyses.

The collection of *C. intybus* hairy roots includes the samples that differ in growth fate, color, the possibility of direct shoots regeneration from hairyroots, biological activity (antioxidant, reducing, and anti-inflammatory), the content of carbohydrates, flavonoids, artemisinin, inulin.

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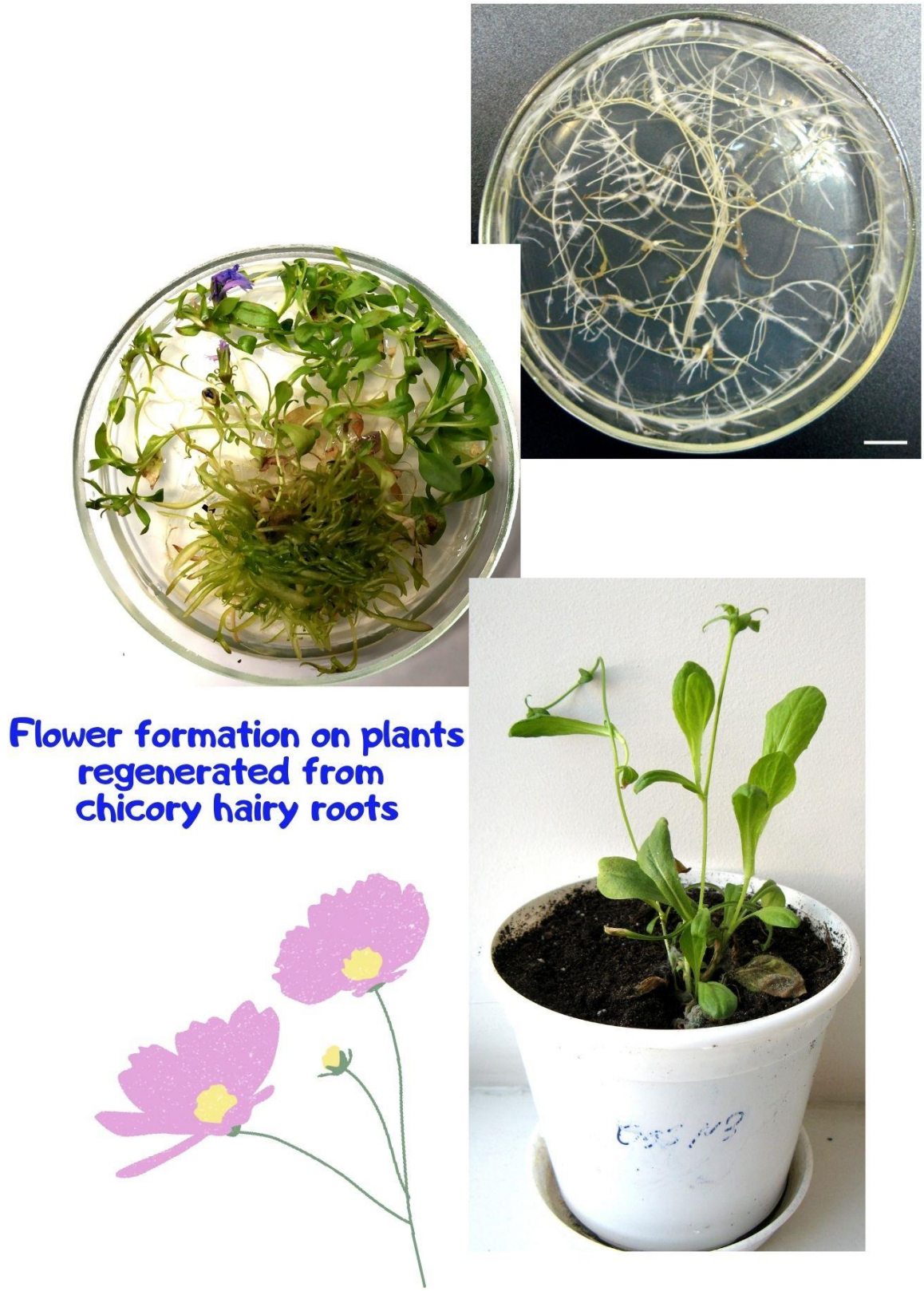

Figure 47 – *Cichorium intybus* hairy roots and regenerated plants

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***Ruta graveolens* L., or rue** plants are native to the Balcan Peninsula region. The plants are cultivated as culinary herbs and as insect repellent. They synthesize coumarins, flavonoids, furanocoumarins, and alkaloids (Oliva et al., 2003). Extracts from *R. graveolens* have been used in the treatment of infections, inflammations, and eczema (Wink, 1998).

Rutin, alkaloids, essential oils, and other chemicals were found in aerial parts and roots (Orlita et al., 2008; Ekiert et al., 2005; Orlanda et al., 2015; Meepagala et al., 2005). Rue extracts possess antimicrobial (Orlanda et al., 2015; Ivanova et al., 2005), antifungal (Meepagala et al., 2005), and analgesic (Cunha et al., 2015) activity. The antioxidant and anti-inflammatory properties of *R. graveolens* were evaluated (Motamed et al., 2014; Raghav et al., 2006). A study of anti-tumor activity demonstrated that rue extracts were found to be cytotoxic for lymphoma and carcinoma cells (Preethi et al., 2006). Etanolic rue extracts showed no toxicity but caused the death of skin melanoma cells (Ghosh et al., 2015). Thus, *R. graveolens* plants could be used as a natural source for medical compound production and possible applications in the pharmaceutical industry.

Hairy root cultures were established after inoculation of hypocotyls with wild *A.rhizogenes* strain LBA 9402 (Sidwa-Gorycka et al., 2009). The authors identified GC and GC-MS coumarins, furanocoumarins, and alkaloids and compared their content with those present in in vitro shoot cultures. The level of pinnarin and rutacultin, bergapten, isopimpinelin. and xanthotoxin was approximately twofold higher in hairy root than in shoot cultures.

Hairy roots of *R. graveolens* were constructed using *A. rhizogenes* strain A4 (Matvieieva et al., 2015c). Transformation frequency (TF) was found to be 2 % and 3 % in the case of internodes and leaves using, respectively. This parameter depended on the type of explant and the time of its cultivation on the medium without cefotaxime. The prolongation of this period has led to TF increasing (Drobot et al, 2016a).

An efficient *R. graveolens* L. shoot regeneration protocol has been developed (Matvieieva, Shakhovsky, 2017). MS media supplemented with 3% sucrose, Kinetin, 6-Benzylaminopurine (BA), and α-Naphthylacetic acid (NAA) growth regulators were used to find out the regeneration ability of intermodal, root, petiole, and leaf explants. The adding BA in concentration 0.5 mg L^{-1} promoted regeneration from petioles, roots, and internodes with 100% frequency. The high shoot regeneration frequency was also observed when the explants were cultivated on the MS basal medium with BA (0.5-1.0 mg L^{-1}) and NAA (0.05-0.5 mg L_{-1}) (Fig. 48).

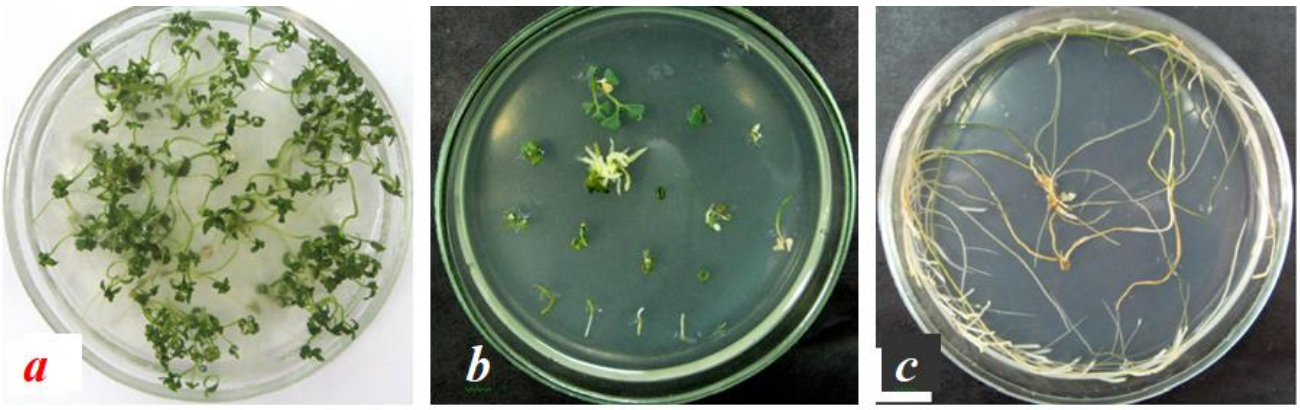

Figure 48 – Initiation of *Ruta graveolens* hairy roots using *A. rhizogenes*: *in vitro* cultivated plants (a), roots formation on the explants (b), hairy roots growth (c)

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***Artemisia balchanorum* Krasch (tarragon lemon)** is a perennial shrub endemic to Turkmenistan, found in mountainous areas. The plant is rich in vitamin C. The citral isolated from the plant is used in pharmaceuticals for vitamin A synthesis. The plants also accumulated an essential oil with a citrus aroma. These plants synthesize sabinene, myrcene, thujone, linalool, nerol, neral, geraniol, and other valuable compounds. We introduced these plants into *in vitro* culture and investigated the possibility of genetic transformation. Transformed roots that had the interferon gene were obtained. The starting plants were obtained from L. Svydenko, Novo-Kakhovskaya Research Station, Ukraine. We also performed the genetic transformation of the plants using *A. rhizogenes* and produced hairy roots with the human interferon-α2b gene (unpublished results, Fig. 49).

***Tragopogon porrifolius* L.,** or **common salsify, oyster plant, vegetable oyster, and Jerusalem star**, belongs to the Asteraceae family. It is an annual or biennial plant. The plants are cultivated for their ornamental flowers and edible roots. *T. porrifolius* ethanol extract showed a high level of antioxidant activity and inhibitory activity for α-glucosidase and α-amylase. The extract exhibited antimicrobial activity in the concentration range of 0.039–2.5 mg/ml and the anticancer effect in the treatment of MDA-MB-231 breast cancer cells (Nuraniye Eruygur et al., 2020). The plants are used in folk medicine (Ozlem et al., 2013). *T. porrifolius* accumulates monounsaturated fatty acids, essential fatty acids, vitamins, and polyphenol components (Formisano et al., 2010; Sareedenchai et al., 2009). The extracts also possessed hepatoprotective activity (Tenkerian et al., 2015).

We transformed the plants using *A. rhizogenes* A4 wild strain and bacteria carried the pCB161 vector with the target human interferon–α2b gene (Fig. 50). In 10-14 days after transformation with the wild strain of *A. rhizogenes* root formation at a frequency of 59.4% was observed. In the case of using *A. rhizogenes* carrying pCB161 vector root formation incidence was 37.5% (Matvieieva, 2012b).

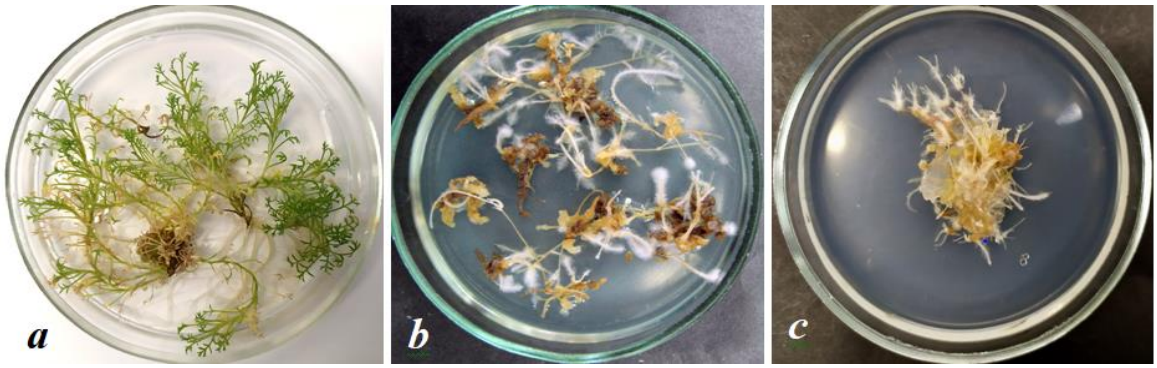

Figure 49 – Initiation of *Artemisia balchanorum* hairy roots: *in vitro* cultivated plants (a), roots formation on the explants (b), hairy roots growth (c)

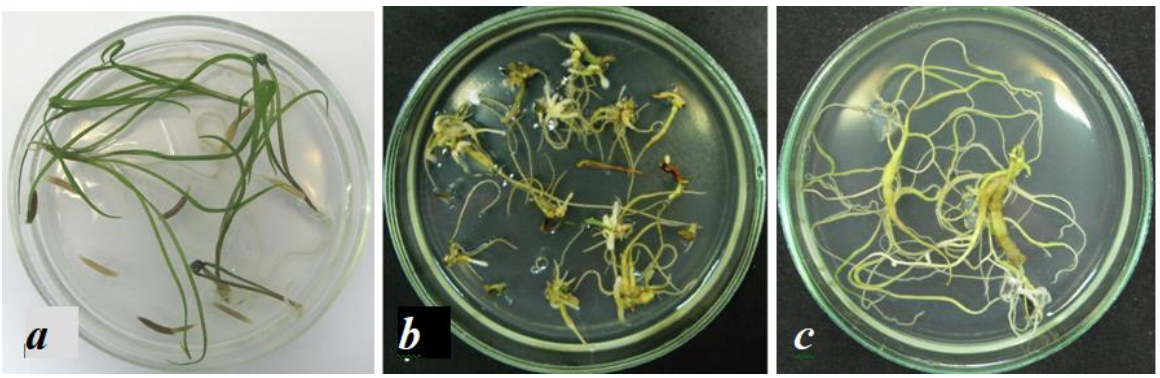

Figure 50 – Initiation of *Tragopogon porrifolius* hairy roots using *A. rhizogenes* (Matvieieva, 2012): *in vitro* cultivated plants (a), roots formation on the explants (b), hairy roots growth (c)

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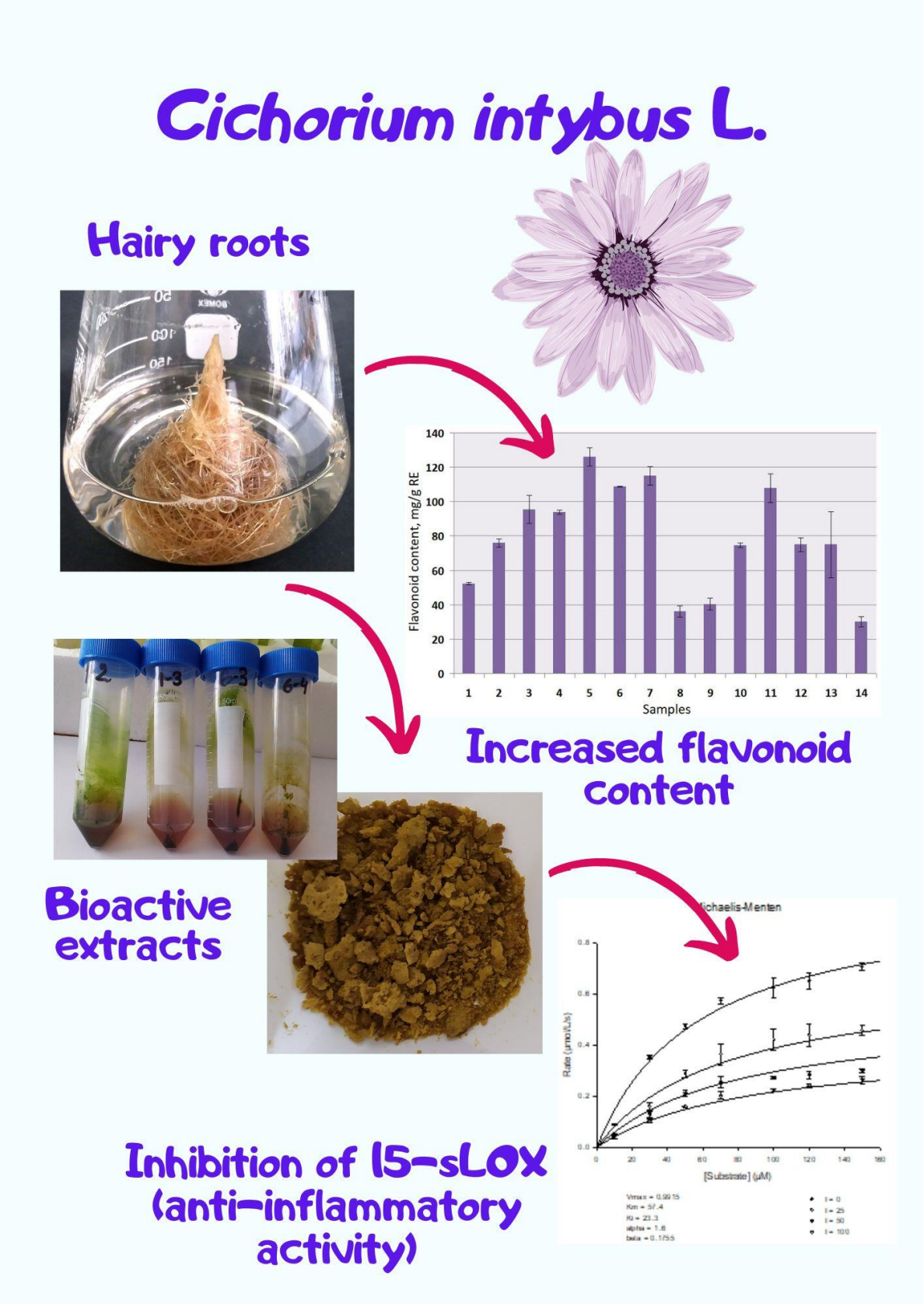
3.16. Bioactivity of hairy roots from the collection of icbge nas of ukraine in diagrams



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Tragopogon porrifolius L,

Antioxidant,
antimicrobial,
anticancer,
hepatoprotective activity.

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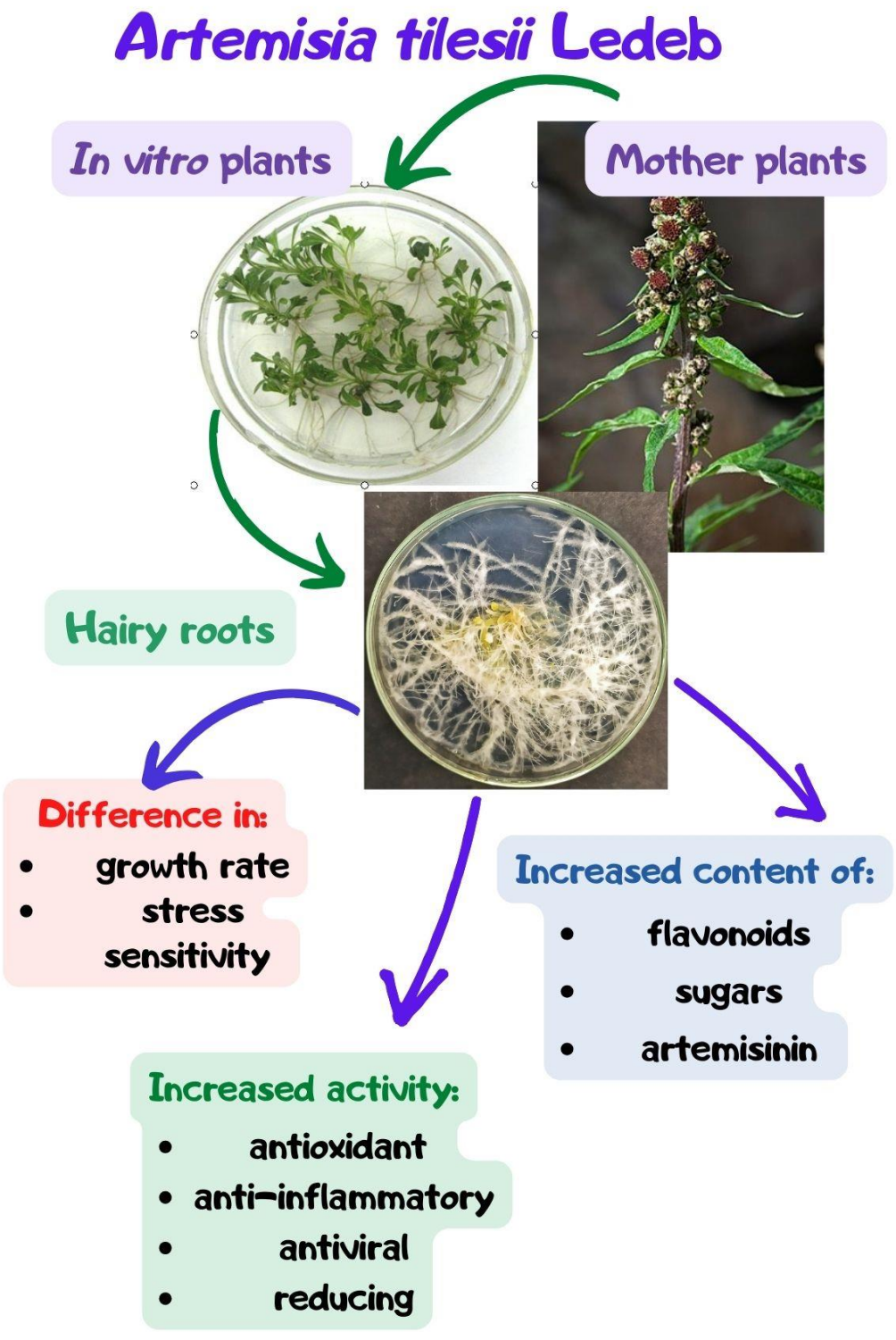

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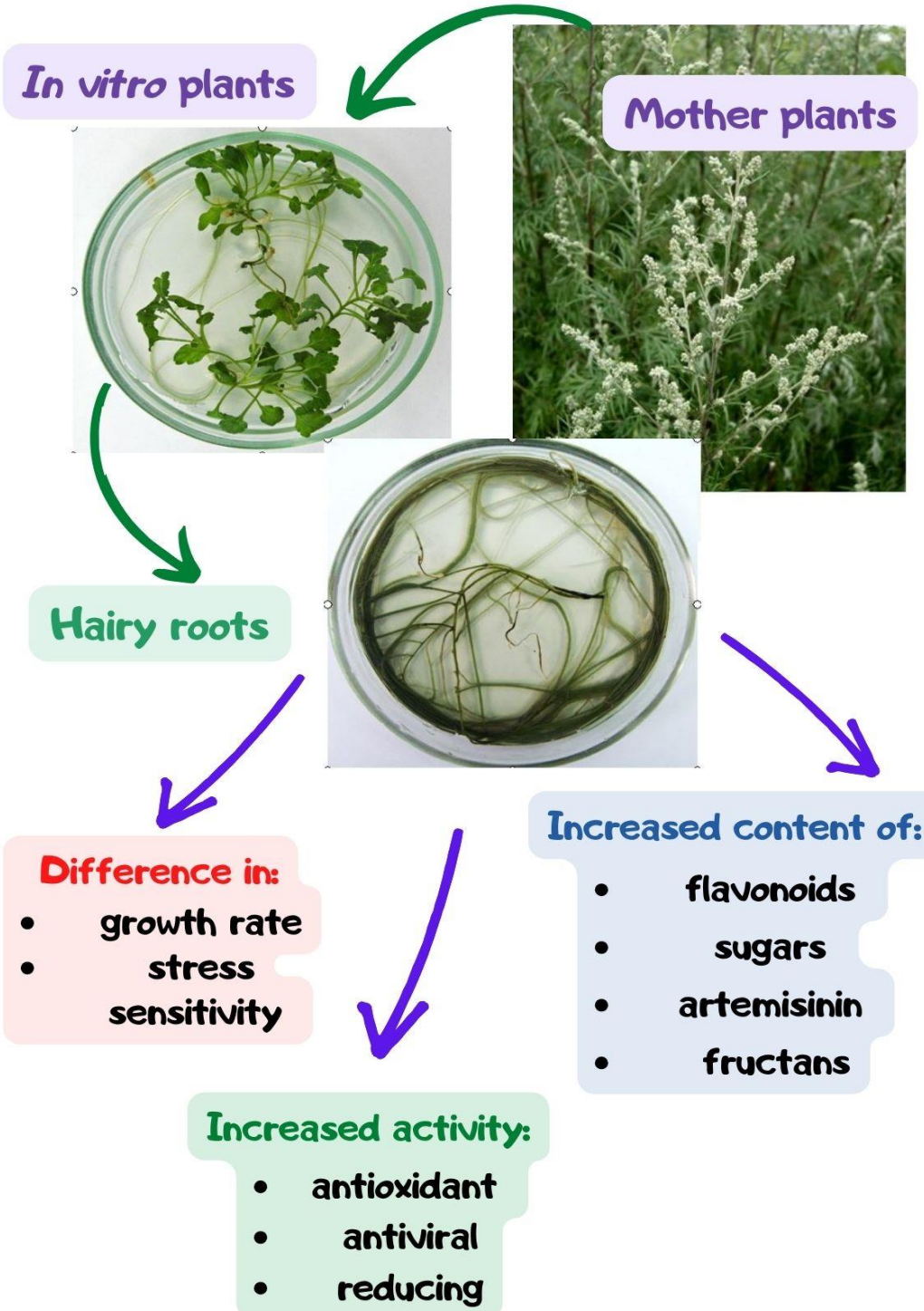
Artemisia vulgaris L.



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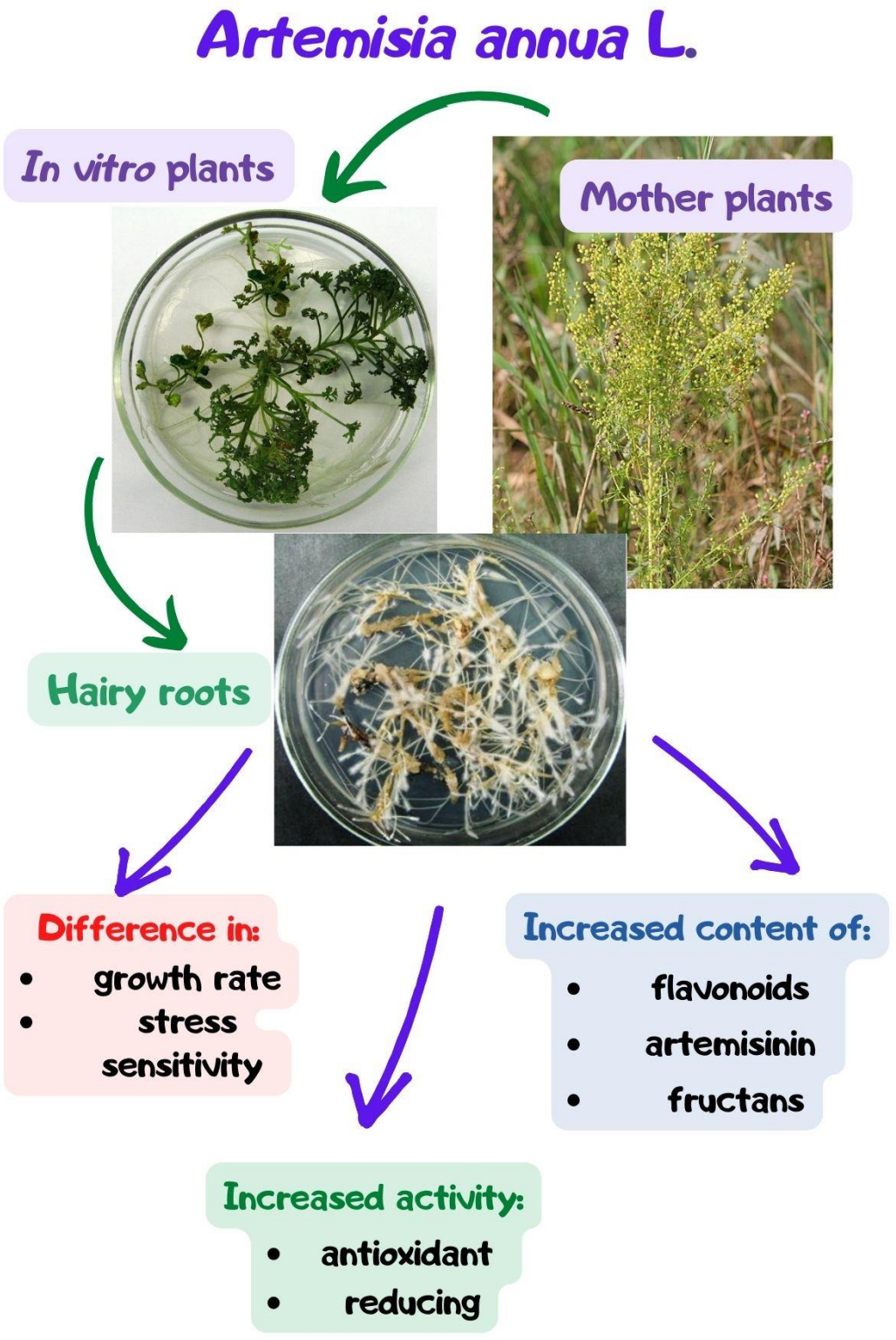

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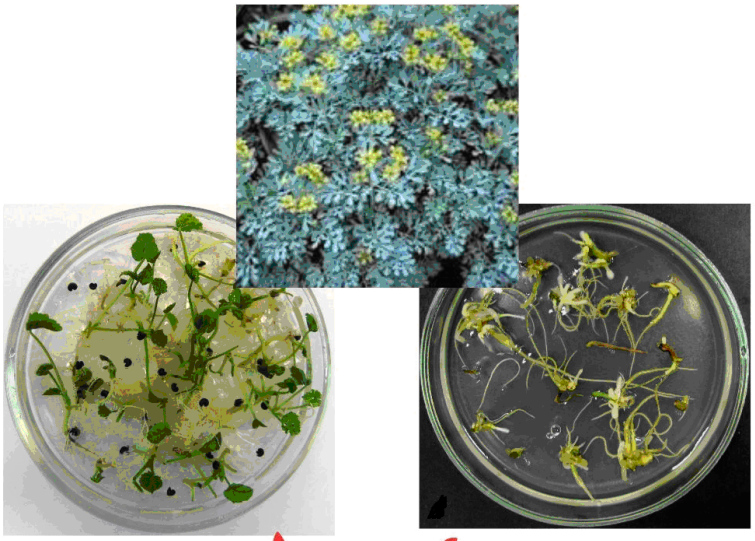
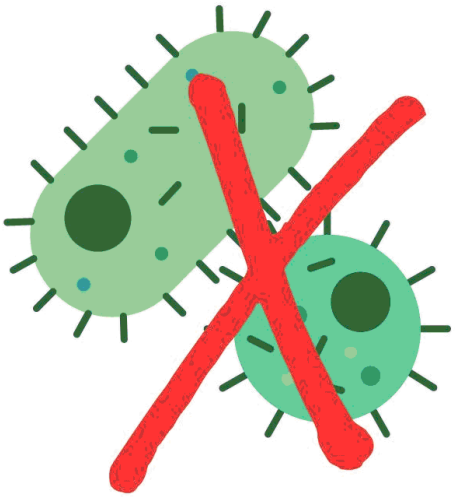
Ruta graveolens L bioactivity





Extracts from the hairy roots of *R. graveolens* and regenerated plants had antimicrobial activity against *Micrococcus luteus*, *Sporosarcina aquimarina*, *Bacillus simplex*, *Kocuria carniphila*, *Bacillus subtilis* subsp. spizizenii, comparable or higher than the extracts from the mother rue plants.

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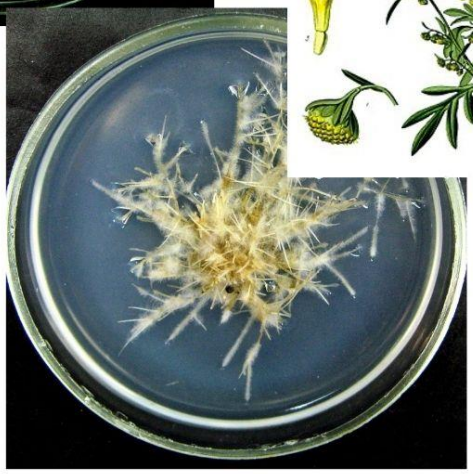
Artemisia absinthium L.

In vitro plants

Mother plants



Hairy roots

Difference in:

- growth rate

Increased content of:

- flavonoids
- polyphenols

Increased activity:

- antioxidant
- reducing

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Artemisia dracunculus L.

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4. CONCLUSIONS

Using knowledge of the natural processes of interaction between plants and bacteria, researchers found the possibility to create new organisms that have improved their qualities, in particular, can synthesize valuable biologically active compounds. Such new organisms, specifically hairy roots, can be used for human benefit.

The book provides detailed information on the synthesis of biologically active compounds in the roots of plants of various species and demonstrates the possibility of increasing their functional potential. We hope this knowledge will help students in their education and open modern horizons of biotechnology for the readers as an advanced science of the 21st century for experts in other fields.

The results of our study for nearly 20 years are presented in this book. They demonstrated the possibility of *Agrobacterium rhizogenes* used for the genetic transformation of different medicinal plants (*Artemisia vulgaris, A. annua, A. dracunculus, A. absinthium, A. tilesii, A. ludoviciana, A. balchanorum, Bidens pilosa, Cichorium intybus, Althaea officinalis, Tragopogon porrifolius, Lactica sativa, Ruta graveolens*) for hairy roots obtaining.

The short time cocultivation of the explants (leaves, internodes, roots) has led to the initiation of the root formation. The bacterial genes (*rol* B and *rol* C) incorporation in plant cells was proved by PCR analysis using primers specific to these genes. The obtained hairy roots were successfully cultivated over a long period and were characterized by significant differences in their morphology and biochemical parameters.

It was studied that the transformation and bacterial gene transfer affect plant cell metabolism. This influence was expressed in changes in the synthesis of various components inherent in the mother plants (flavonoids, polyphenols, polysaccharides, etc.). It has been proved that it is possible to influence this metabolism by changing the cultivation conditions, in particular, varying the composition of the nutrient medium.

Such studies on a large number of collection samples (about 100 samples) and using the hairy roots of plants of various species have undeniably proved the significant influence of *Agrobacterium rhizogenes* on the metabolism and functioning of plant cells. Such fundamental research can become the basis for the recommendations regarding their practical application. It was proved by obtaining hairy roots of medicinal plants with high (much more than the control) levels of synthesis of valuable compounds that have antioxidant, antiradical, DNA-protective, and antiviral properties. Based on previously developed methods of hairy root cultivation in bioreactors, it is possible to assert prominent prospects for hairy roots of medicinal plants as natural biofactories for valuable compound production. They can be used in the pharmaceutical industry to develop safe drugs from natural plant raw materials.

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