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Role of Biotechnology for Protection of Endangered Medicinal Plants

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1. Introduction

The last two centuries of industrialization, urbanization and changes in land use converting agricultural and natural areas to artificial surface have led to European plants being considered amongst the most threatened in the world. In some countries, more than two-thirds of the existing habitat types are considered endangered. Human activity is the primary cause of risk for 83% of endangered plant species. Habitat destruction and loss are also a problem because they lead to the fragmentation of the remaining habitat resulting in further isolation of plant population [1]. From another side during the last 10 years an intense interest has emerged in "nutraceuticals" (or "functional foods") in which phytochemical constituents can have long-term health promoting or medicinal qualities. Although the distinction between medicinal plants and nutraceuticals can sometimes be vague, a primary characteristic of the latter is that nutraceuticals have a nutritional role in the diet and the benefits to health may arise from long-term use as foods (i.e. chemoprevention) [2]. In contrast, many medicinal plants possess specific medicinal benefits without serving a nutritional role in the human diet and may be used in response to specific health problems over short- or long-term intervals [3].

There is indisputable interest towards traditional and alternative medicine world-wide [4] and at the same time an increasing application of herbs in medical practices, reported by World Health Organization (WHO) [5]. Nowadays the centuries-old tradition of medicinal plants application has turned into a highly profitable business on the world market. Numerous herbal products have been released like patented medical goods, food additives, herbal teas, extracts, essential oils, etc [6 - 9].

There is an expansion of the market of herbs and herbs based medical preparations all over the world. The income a decade ago in the North American market for sales of medicinal plants has climbed to about \$3 billion/year [10]. In South America, Brazil is outstanding with 160

millions USD for 2007 while in Asia, China is at the leading trade position with 14 billions USD for 2005, etc. [11]. Similar increase was observed in Western Europe with 6 billion USD income for a period of two years from 2003 to 2004. The sales increased in Czech Republic by 22 % from 1999 to 2001 and jumped twice in Bulgaria [12].

Medicinal plants are precious part of the world flora. More than 80 000 species out of the 2 500 000 higher plants on Earth are reported to have at least some medicinal value and around 5 000 species have specific therapeutic value. The contemporary phytotherapy and the modern allopathic medicine use raw materials from more than 50 000 plant species [13]. About two thirds of these fifty thousand plants utilized in the pharmacological industry are harvested from nature [14]. Small portions like 10%-20% of the plants used for remedies preparations are cultivated in fields or under controlled conditions [15]. Ages-old exploitation of the natural resources and the dramatically increased interest are a real thread for the biological diversity. Bad harvesting management and insufficient cultivation practices may lead to extinction of endangered species or to destruction of natural resources. Science has already recorded diminishing natural populations, lost in the genetic diversity, local extinction of many species and/or degeneration of their natural habitats [16]. This alarming situation is raising the questions about special efforts which should be paid both to protection of the plant populations and to up-to-date knowledge concerning more reasonable and effective utilization of these plants [12].

Bulgaria as a country with a rich and diverse flora (comprising of 7 835 species) and with old traditions in herbs' use faces the same global problems. One of the most serious challenges is the control and the limitation of the expanding gathering of endangered medicinal plants [17]. The Biodiversity Act covers *Sideritis scardica* (mursala tea), *Alchemilla vulgaris*, *Acorus calamus*, *Rhodiola rosea*, *Leucosium aestivum*, *Gentiana* sp., *Glycyrrhiza glabra*, *Ruta graveolens*, and some medicinal plants under special rules of protection and use e.g. *Inula helenium*, *Carlina acanthifolia*, *Berberis vulgaris*, *Rhamnus frangula*, *Rubia tinctorum*, *Atropa belladonna*, *Origanum heracleoticum* etc. More than 750 herbs are used in the folk medicine while 150 - 250 are used in the official medicine and can be found at the market [18 - 20]. A considerable number of the wild species are rare, endangered or under protection [21, 22], and 12.8% are endemics [23]. Recently 120 herbs have been traditionally collected from their natural populations, 47 are under protection, 38 are included in the Red Data Book of Bulgaria, 60 have been cultivated, 35 are main industrial crops [24]. Bulgarian medicinal plants are famous for their high content of biologically active substances. Their high value qualities are due to the unique combinations of specific soil and climatic conditions in the different sites of the country [25]. Bulgaria is the European leader in herbs export and occupies the 8th world position with trade in 40 countries all over the world. The greatest export of 50% is to Germany being 3 600 tones in 1991 and doubling to 6 000 tones in 2 000 [8]. Spain, Italy, France, Austria and USA are also major trade partners. The export is increasing steadily from 6 - 7 t in 1992 to 12 tones in 2000 - 2003, to 15 - 17 000 tones in 2007 [22, 26 - 28,]. These amounts represented about 70% - 80% out of all harvested and processed medicinal plants in Bulgaria [27]. The bigger number of these species is wild growing [29] but recently cultivation in fields has been applied as a measure to protect

medicinal plants included in the list of protected species. At present about 20% of the medicinal plants are cultivated, but this share comprises about 40% of the export [24].

Worldwide the constant expansion of herbs' trade, the insufficient cultivation fields, and the bad management of harvesting and overharvesting have led to exhaustion of the natural resources and reduction of the biodiversity. According to the data of the Food and Agricultural Organization (FAO) at the United Nations annually the flora bares irretrievable losses which destroy the natural resources and the ecological equilibrium [30]. Four thousand to 10 000 medicinal species were endangered of disappearing at the beginning of this century [14]. To stop the violence against nature, efforts should be directed both to preservation of the plant populations and to elevating the level of knowledge for sustainable utilization of these plants in traditional, alternative, and allopathic medicine [12].

This great issue is in the focus of science which offers different decisions to solve the global problem. Cultivation of the valuable species in experimental conditions is one of the approaches. The latter refers to application of classical methods for multiplication by cuttings, bulbs, and so forth, as well as by biotechnological methods of *in vitro* cultures and clonal propagation for production of enormous number of identical plants. The micropropagation is considered to have the greatest commercial and iconomical importance for the rapid propagation and *ex situ* conservation of rare, endemic, and endangered medicinal plants [31 - 34]. Except for clonal multiplication and maintaining the genetic structure biotechnology is powerful for modifying genetic information and gene expression to obtain new valuable compounds with new properties or with increased amounts [35 - 37]. Micropropagation, cell and callus cultures, metabolic engineering and genetic manipulations are especially appropriate for species which are difficult to propagate *in vivo* [36].

In Bulgaria quite successful investigations have been performed for *in vitro* clonal multiplication of valuable, endemic, rare and endangered medicinal species: *Rhodiola rosea*, *Gentiana lutea*, *Sideritis scardica*, *Pancratium maritimum*, *Scabiosa argentea*, *Cionura erecta*, *Jurinea albicaulis* subsp. *kilaea*, *Peucedanum arenarium*, *Linum tauricum* subsp. *bulgaricum*, *Aurinia uechtritiziana*, *Silene thymifolia*, *Glaucium flavum*, *Stachys maritima*, *Astrodaucus littoralis*, *Otanthus maritimus*, *Plantago arenaria*, *Verbascum purpureum*, *Alchemilla sps*, etc. [38 - 44]

More than 2 000 different species are used in Europe for production of medicinal and other herbal preparations. Seventy percents of these species are growing in wild nature [17, 29] with already limiting resources which demands search for alternative methods friendly to nature. Biotechnological methods seem appropriate ones with their potential for multiplication, selection and protection of medicinal plants. In this respect biotechnological approaches are convenient for use of cells, tissue, organs or entire organism which grow and develop in *in vitro* controlled conditions, and can be subjected to *in vitro* and genetic manipulations [33] to obtain desired substances [45]. These methods are especially appropriate and reasonable to apply when the targeted species have high economical or trade value, or plant resources are limited concerning the availability of wild area or good healthy plants, or when the plants are difficult to grow [46, 47].

In vitro cultivation may be directed to development of different systems depending on the practical needs. At present production of a large number of identical plants by clonal micropropagation is the most prominent one. Complex and integrated approaches for cultivation of plant systems may be the basis for future development of new, effective, safe and high quality products. These scientific achievements might be used for the establishment of *ex situ* and *in vitro* collections, multiplication of desired species and to obtain raw material for the pharmaceutical and cosmetic industries [48]. *In vitro* technologies offer some or most of the following advantages: easier extractions and purification of valuable substances from temporary sources; new products which may not be found in nature; absence of various environmental and seasonal effects, automation, better control of the biosynthetic pathways and flexibility in obtaining desired product; shorter production cycles and cheaper less costly products. Here should also be mentioned the potential of the sophisticated techniques of genetic engineering, which might be applied respecting the rules of contained use [33; 47]. At present the methods of plant cell and tissue cultures have found many proper sites for application in the medicinal plants utilization. The achieved results and the confidence for further success drive the efforts for wider application of plant biotechnologies in more spheres concerning medicinal plants [37].

2. Essence of *in vitro* culture

Plant cell methods and techniques were initially used in fundamental scientific investigations at the beginning of their development in the early 60-ties of the last century. Plant biotechnology is based on the totipotence of the plant cell [35; 49]. This process of *de novo* reconstruction of an organism from a cell in differentiated stage is highly linked to the process of dedifferentiation when the cell is returning back to its early embryogenic/meristematic stage. In this stage cells undergo division and may form nondifferentiated callus tissue or may redifferentiate to form new tissue, organs and an entire organism. Morphogenesis *in vitro* is realized via two major pathways: (i) organogenesis when a group of cells is involved for *de novo* formation of organs and (ii) somatic embryogenesis when the new organism is initiated from a single cell.

3. Micropropagation

Micropropagation is a vegetative propagation of the plants *in vitro* conditions (in glass vessels under controlled conditions) leading to development of numerous plants from the excised tissue and reproducing the genetic potential of the initial donor plant.

Usually tissues containing meristematic cells are used for induction of axillary or adventitious shoots but induction of somatic embryos can be achieved from differentiated cells as well.

Micropropagation is used routinely for many species to obtain a large number of plants with high quality. It is widely applied to agricultural plants, vegetable and ornamental species, and

in some less extent to plantation crops. One of the substantial advantages of micropropagation over traditional clonal propagation is the potential of combining rapid large-scale propagation of new genotypes, the use of small amounts of original germplasm (particularly at the early breeding and/or transformation stage, when only a few plants are available), and the generation of pathogen-free propagules. [50]. Compared to the other spheres of *in vitro* technologies clonal propagation has proved the greatest economical and market importance in industry including pharmaceutical industry which needs for raw material from the medicinal plants is increasing constantly. It offers faster and alternative way for production of raw material and from another side overcoming the problems arising from the limited natural resources.

At present, there is a long list of research groups worldwide investigating hundreds of medicinal species. Various success procedures and recipes for many of these species have been developed. However, there is not a universal protocol applicable to each species, ecotype, and explant tissue. From another side all these continuous tedious studies on the standardization of explant sources, media composition and physical state, environmental conditions and acclimatization of *in vitro* plants have accumulated information, continuously enriched, which is a good basis for elaboration of successful protocols for more species. Wider practical application of micropropagation depends on reduction of costs so that it can become competitive with seed production or traditional vegetative propagation methods (e.g., cuttings, tubers and bulbs, grafting) [50].

4. Metabolic engineering and biotransformation

The plant cell culture systems have potential for commercial exploitation of secondary metabolites. Similar to the fermentation industry using microorganisms and their enzymes [35, 51, 52] to obtain a desired product plant cells are able to biotransform a suitable substrate compound to the desired product. The latter can be obtained as well by addition of a precursor (a particular compound) into the culture medium of plant cells. In the process of biotransformation, the physicochemical and biological properties of some natural products can be modified [53]. Thus, biotransformation and its ability to release products into the cells or out of them provide an alternative method of supplying valuable natural products that occur in nature at low levels. Generally, the plant products of commercial interest are secondary metabolites, which in turn belong to three main categories: essential oils, glycosides and alkaloids [51]. Plant cell cultures as biotransformation systems have been highlighted for production of pharmaceuticals but other uses have also been suggested as new route for synthesis, for products from plants difficult to grow, or in short supply, as a source of novel chemicals. It is expected that the use, production of market price and structure would bring some of the other compounds to a commercial scale more rapidly and *in vitro* culture products may see further commercialization [54]. The application of molecular biology techniques to produce transgenic cells and to effect the expression and regulation of biosynthetic pathways is also a significant step towards making *in vitro* cultures more generally applicable to the commercial production of secondary metabolites [54]. However, because of the complex and incompletely understood nature of plant cells growing in *in vitro* cultures, case-by-case studies

have been used to explain the problems occurring in the production of secondary metabolites from cultured plant cells.

Genetic manipulations (direct and indirect genetic transformation) are other different approaches to increase the content biological active substances in plants. Genetic engineering covers a complex of methods and techniques applied to the genome in order to modify it to obtain cells and organisms with improved qualities or possessing desired traits. These might refer to better yield or resistance, as well as, to higher metabolite production or synthesis of valuable biologically active substances [55]. Gene transfer may be direct when isolated desired DNA fragments are inserted into the cell most often by electrical field or adhesion. This method is less used in medicinal plants. Indirect genetic transformation of plants uses DNA vectors naturally presenting in plant pathogens to transfer the isolated genes of interest and to trigger special metabolic pathways [56]. *Agrobacterium rhizogenes* induces formation of "hairs" at the roots of dicotyledonous plants. Genetically modified "hairy" roots produce new substances, which very often are in low content. Hairy roots are characterized with genetic stability and are potential highly productive source for valuable secondary metabolites necessary for the pharmaceutical industry [57, 58]. Manipulations and optimization of the productivity of the transformed hairy roots are usually the same as for the other systems for *in vitro* cultivation [59]. They also depend on the species, the ecotype, the explant, the nutrient media, cultivation conditions, etc [60].

All these application of the principles of plant cell division and regeneration to practical plant propagation and further manipulations could be possible if there are reliable *in vitro* cultures, which efficiency depends on many various factors.

5. Factors influencing cell growth *in vitro*

The ability of the plant cell to realize its totipotency is influenced in greatest extent by the genotype, mother/donor plant, explant, and growth regulators what was confirmed by the tedious empirical work of *in vitro* investigations [61, 62]. Here, some of the specific and most important requirements will be mentioned in order of understanding the efforts and originality of some ideas when establishing *in vitro* cultures of medicinal plants.

Genotypes. Morphogenetic potential of excised tissue subjected to cultivation *in vitro* is in strong dependence of the genotype [63]. Genetically plants demonstrate different organogenic abilities, which were observed for all plants groups including medicinal plants [64 - 72]. Some of the species (like tobacco and carrot) are easy to initiate in *in vitro* cultures while others are more difficult - recalcitrant (cereals, grain legumes, bulbous plants). Many of the wild species like most of the medicinal plants and especially those producing phenols are more difficult or extremely difficult to handle.

Donor plant. The donor plant should be healthy, in the first stages of its intensive growth, not in dormancy. Rhizomes and bulbs usually need pretreatment with low or high temperatures for different periods of time [35, 73].

Explant. The explant type might determine the organogenesis potential and the genetical stability of the clonal material. Physiological age of the explant is also crucial. Immature organs and differentiated cells excised from stem tips, axillary buds, embryos and other meristematic tissues are the most appropriate [35, 62, 73]. However, despite the development of cell and molecular biology the limits still exist in receiving easy information about the genetic, epigenetic and physiological status of the explant. Empirical approach is the most common to specify the chemical and physical stimuli triggering cell totipotency.

Nutrient media. Although more than 50 different media formulations have been used for the *in vitro* culture of tissues of various plant species the formulation described by Murashige and Skoog (MS medium) [74] is the most commonly used, often with relatively minor changes. Other famous media are those of Gamborg [75; 76], Huang and Murashige [77] Nischt and Nischt etc. The nutrient medium usually consists of all the essential macro- and micro salts, vitamins, plant growth regulators, a carbohydrate, and some other organic substances if necessary [62].

Plant growth regulators. Plant growth regulators, including the phytohormones, are essential for cell dedifferentiation, division and redifferentiation leading to callus tissue and organ formation. The auxins and cytokinins are the most important for *in vitro* development and morphogenesis. However, the most appropriate plant regulators and their concentrations in the nutrient media depend on the genotype, explants type and the donor plant physiological status. Hence, numerous combinations could be designed and the optimal ones are validated empirically. All that creates the difficulties of the experimental work, which is dedicated to find the balance between the factors determining reliable *in vitro* development.

Cytokinins. Different groups of cytokinins might be used but the most efficient ones for induction of organogenesis and a large number of buds are the natural cytokinins (zeatin and kinetin) or the synthetic ones - 6-benzylaminopurine (benzyl adenine (BA, BAP), 6- γ -(dimethylallyl-amino)-purine (2iP) and thidiazuron (TDZ).

Auxins. The auxins also are obtained from natural plant materials like indolyl-3-acetic acid (IAA), indole 3-butyric acid (IBA), α -naphthyl acetic acid (NAA) or are chemically produced like 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), picloram, etc. The auxins have a wide spectrum of effects on different processes of plant development and morphogenesis. Depending on their chemical structure and concentration, they induce or inhibit cell division, stimulate callus or root formation.

Gibberellins. The group of gibberellins includes more than 80 compounds, which stimulate cell division and elongation. The most commonly used one is gibberellic acid (GA₃).

Vitamins and supplements. Growth regulatory functions are attributed to some of the vitamins B group – thiamine (B1), niacin (vit B₃, nicotinic acid, vitamin PP), piridoxin (vit B₆), which in fact are the most popular for *in vitro* recipes. Supplements like yeast extract, coconut milk, maize extract and some other might effect tissue growth and bud development.

The best morphogenesis could be achieved when the optimal balance between the effect of genotype, explant and growth regulators is identified.

6. Rooting, acclimatization and adaptation

The processes of root formation and adaptation have their specific requirements and not all of the quoted cases of organogenesis, embryogenesis, regeneration are followed by rhizogenesis and adaptation. These processes depend on the genotype and in most of the cases on the ecotype of the species [62], whereas the necessary culture conditions are chosen in an empirical way. The reduction of the sucrose from 2 % - 3% to 1% - 0.5% stimulates root induction and formation. Acclimatization of the obtained *in vitro* plants is a critical moment for establishment of good protocol for micropropagation. Adaptation of plants in greenhouse, field or in the nature is another delicate and difficult stage. Usually in *in vitro* conditions, regenerants formed well-developed root system. However, they quickly lose their turgor after transfer to soil. Their leaves withered and dried. These plants underwent stress due to the changes in humidity and culture medium.

7. *In vitro* cultures conditions

The light, temperature and air humidity are important parameters for *in vitro* cultivation of the plant cells and tissues. The light is one of the important factors for morphogenetic process like bud and shoots formation, root induction and somatic embryogenesis. Light spectrum and intensity as well as the photoperiod are very important for successful cultivation [78]. The recommended temperature in the cultivation rooms or phytotron chamber is about 23-25 °C but the cultures of tropical species require higher temperature (27-30°C), while arctic plants cultures – lower (18-21° C).

Efficient protocols for *in vitro* propagation (plant cloning) were established for a long list of medicinal plants like *Panax ginseng* [79, 80], *Aloe vera* [81], *Angelica sinensis*, *Gentiana davidii* [82], *Chlorophytum borivillianum* [83, - 86], *Tylophora indica* [87, 88], *Catharanthus roseus* [89], *Holostemma ada-kodien* and *Ipomoea mauritiana* [90], *Saussurea involucreata* [91], *Kniphofia leucocephala* [92], *Podophyllum hexandrum* [93], *Saussurea obvallata* [94], *Ceropegia candellabrum* [95], *Syzygium alternifolium* [96], *Chlorophytum arundinaceum* [97], *Rotula aquatica* [98, 99], etc.

Establishment of micropropagation system is a base for conservation of the species and for protection of the gene fund, as well as for studies of valuable substances in important medicinal plants. Different strategies are developed as well for establishment of cell cultures aiming at production of biologically active compounds. These systems could be used for large scale cultivation of plant cells for obtaining of secondary metabolites. These methods are reliable and give possibility for continuous supply of raw materials for production of natural products [45, 82].

8. *In vitro* cultures and application of biotechnology in *Gentiana*, *Leucojum* and *Rhodiola*

In this chapter a small part of the successful *in vitro* research in medicinal plants and the application of “green biotechnology” methods for protection of endangered species will be illustrated by examples from the investigations in the genera *Gentiana*, *Leucojum* and *Rhodiola*. These groups of medicinal plants were chosen because the three of them are with outstanding importance for the pharmaceutical and nutraceutical industries. The species belonging to them are worshiped for their multiple beneficial health effects and have been used for thousands of years in folk medicine all over the world. However, their distribution is at different parts of the Earth – *Gentians* are the most widely spread in various climatic zones, *Rhodiola* covers less territory, predominantly in the cold regions in north and high mountains, while *Leucojum* can be found in limited warm and south regions in Europe. Many species from *Gentiana*, *Rhodiola* and *Leucojum* genera can be found in Bulgaria but most of them are endangered and included in the Red Book like *Gentiana lutea*, *Rhodiola rosea* and *Leucojum aestivum*. In world scale level of protection – *Leucojum spp* are in the list of the most threatend with heavy measures of restriction. Nearly all *Gentiana* species are endangered while many *Rhodiola* species are under special regime of use. However, one and the same *Rhodiola* species may be close to extinction in one country but widely spread (even as a weed) in another country. Another consideration of ours is the ability of the plants from these genera to be cultivated in field what was possible for *Gentiana*, partially for *Rhodiola* and not possible for *Leucojum*. Described here examples illustrate different levels of development of *in vitro* cultures and application of biotechnology to the three chosen groups of herbs. Development of *in vitro* cultures started about 40 years ago in *Gentiana*, 25 years ago in *Rhodiola* and 20 years ago in *Leucojum*.

The most intensive *in vitro* research was carried in *Gentiana* obtaining all kinds of *in vitro* cultures, including somatic embryo cultures, with success in cryopreservation, in biotransformation and genetic metabolic engineering. *Rhodiola* occupies a middle position with considerable success in callus, suspension and micropropagation systems. Cultivation in bioreactors, biotransformation and genetic transformation were successful.

Leucojum seems to be the most difficult, though protocols for clonal propagation have been established and gene bank *in vitro* has been reported (in Bulgaria). Callus, suspension and organogenic cultures could be obtained and growth in bioreactors with possibilities for biotransformation and even genetic transformation (though at this stage without synthesis of galanthamine).

Bulgaria is a pioneer in *Leucojum aestivum* biotechnology. It is in the frontiers of micropropagation of *Rhodiola rosea* and has less investigation in *Gentiana in vitro* cultures.

Genus *Gentiana* belongs to family *Gentianaceae* and is a group of medicinal plants of special interest. It is a large genus comprising of about 400 species widely distributed in the mountain areas of temperate zones [100], including Central and South Europe. Most of the species are interesting to horticulture for their beautiful and attractive flowers but they have more

important medicinal value, which is due to the production of secondary metabolites in their roots (*Radix Gentianae*). The most efficient ones are the bitter secoiridoid glucosides (gentiopicroside, amarogentin), xanthones, di- and trisaccharides, pyridine alkaloids [66, 101]. Traditionally, the pharmaceutical industry largely depends on wild sources exploiting intensively the natural areals. The annual drug demands have been much higher than the production from wild sources [66]. At the same time many gentians are either difficult to grow outside their wild habitat or their cultivation (if possible) proved to be not economic. Continuous collection of plant material from natural habitats has led to the depletion of *Gentiana* population and many representatives of the genus are protected by law. Some of the gentians having the status of endangered species, for example, are: *Gentiana lutea* L. - included in the Red Book of Bulgaria and of other European and world countries [66]; *Gentiana kurroo* Royle - close to extinction and legally protected by law [102]; *Gentiana dinarica* Beck - a rare and endangered species of the Balkan Dinaric Alps; *Gentiana asclepiadea* L. - distributed in South and Central Europe, *Gentiana triflora*, *Gentiana punctata*, *Gentiana pneumonanthea* - under protection of its progressively decreasing habitats; *Gentiana dahurica* Fisch - with exhausted natural resources though this species could be cultivated in some areas of the northwest of China; *Gentiana straminea* Maxim an endangered medicinal plant in the Qinghai-Tibet Plateau [103]. Due to problems with germination of seeds in *in vivo* conditions as well as the high variability of generatively propagated plants these species have attracted the attention of scientists being aware of the potential of biotechnology. The genetic variability of endemic or endangered species is usually very low and methods (like *in vitro* micropropagation) of conservation and restoration of natural resources have been given much attention in the last years. Despite the remarkable success of the tedious and wide investigations worldwide *in vitro* cultures of *Gentiana* species proved to be very difficult to achieve because of their low natural capacity of regeneration which was manifested in the multiplication *in vitro*, too [104].

First investigations on establishment of *in vitro* cultures of *Gentiana* were reported a quarter of century ago. Wesolowska et al. [105] succeeded in induction of callogenesis in *G. punctata* and *G. panonica* and of organogenesis and rhizogenesis in *G. cruciata* and *G. purpurea*. Authors observed that regenerated plants synthesize secoiridoids, which could not be found in the wild plants. This raised the hopes for the application of biotechnology techniques to other species of the *Gentiana* genus. The next decade the scientists explored the basic factors and plant requirements for establishment of *in vitro* cultures and micropropagation in various gentians. Different explants were tested for development of efficient regeneration schemes.

Using shoots and node fragments as explants, regeneration systems of *Gentiana scabra* var *buergeri* [106], *Gentiana kurroo* [107], *Gentiana cerina*, *Gentiana corymbifera* [65], *Gentiana punctata* [108], *Gentiana triflora* [109, 110] and *Gentiana ligularia* [111] were established. Stem segments with meristem tissue were appropriate explants to initiate tissue cultures and to induce formation of shoots *de novo* in four other species of *Gentiana*: *G. lutea*, *G. cruciata*, *G. acaulus* and *G. purpurea* [66]. Different explants (shoot tips, lateral green buds, and root segments) were tested in *Gentiana lutea* [112]. Leaf explants were used as well to induce shoots of *Gentiana macrophylla* [113] and *G. kurroo* Royle [107, 114]. Vinterhalter et al. [115] micropropagated *Gentiana dinarica* Beck using axillary buds as explants.

Seeds in different stage of maturity were object of quite strong interest as an initial plant material for *in vitro* cultures. Considerably high germination of 54 % was achieved when seeds of *G. corymbifera* were cultured on a Murashige and Skoog (MS) medium containing 100 mg/l gibberellic acid (GA₃) for 70 days. In the absence of GA₃ germination did not exceed 5% [65].

Immature seeds in different stages of ripening were tested in order to find out the most suitable initial material to obtain *in vitro* cultures and multiplication of *Gentiana lutea*. Despite the addition of 0.5 mg/l of gibberellic acid to the MS medium, the average germination was quite low 21% [112]. Seedlings from immature and mature seeds of *Gentiana pneumonanthe* and *Gentiana punctata* were also chosen as initial material to excise shoot tips and one-nodal cuttings for induction of organogenesis and further clonal propagation [104, 116]. Petrova et al. [40] studied the possibility for micropropagation of Bulgarian ecotype of *Gentiana lutea* using stem segments with two leaves and apical or axillary buds excised from mature seeds germinated *in vitro* (Figure 1). To increase the germination seeds were treated with 0.03% GA₃ for 24 hours. Some of the seeds were mechanically scarified in the micropile region. Germination was initiated on three variants of nutrient media based on MS and different concentration from 25 to 100 mg/l of GA₃. In these investigations, GA₃ and scarification stimulate *G. lutea* seed germination. Only 20 % of the non-scarified and 33.33 % of the scarified seeds germinated on the control medium. Gibberellic acid in concentration of 50 mg/l proved to have optimum effect resulting in 42.5 % germination for the nonscarified seeds and 60 % for the scarified ones. Lower and higher levels of GA₃ stimulated in a less extend the seed germination but the response to GA₃ of the scarified seeds was stronger than that of the non-scarified ones.

In vitro response is determined, as mentioned before, not only by the explant type but by the media composition as well and by the effect of the plant growth regulators on the dedifferentiation and redifferentiation processes undergoing in the explants cultured *in vitro*. Many reports, especially at the beginning of the *in vitro* investigations of gentians, pointed out that the cytokinin benzyl aminopurine BAP (or benzyl adenine BA) and the auxines indolacetic acid (IAA) or naphthalic acid (NAA) were the best plant growth regulators for induction of organogenesis and regeneration of plants which allowed establishment of a micropropagation schemes. Among the numerous examples, some of them were mentioned below as an illustration.

The initial results of Sharma et al. [107] were very promising reporting fifteen-fold shoot multiplication of *Gentiana kurroo*, which was obtained every 6 weeks on Murashige and Skoog's medium (MS) containing 8.9 µM benzyladenine and 1.1 µM 1-naphthaleneacetic acid. The efficiency of these plant growth regulators were confirmed in the experiments with other species. Optimal shoot multiplication of *G. dinarica* was achieved on MS medium enriched with 1.0 mg/l BA and 0.1 mg/l NAA [115]. The ideal medium for adventitious buds formation and for differentiation of calli contained 0.6 mg/l BA and 0.1 mg/l NAA [117] while the ideal medium for induction of calli from tender stems of *Gentiana scabra* was by substitution of NAA with 2,4 D in concentrations of 1.0-1.5 mg/l at the background of the same cytokinin –BA at lower concentration of 0.2 mg/l.

Momcilovic et al. [66] observed that the optimal concentrations of the two plant hormones BAP and IAA were slightly different in the four investigated species *Gentiana acaulis* L., *G. crucia-*

ta L., *G. lutea* L. and *G. purpurea* after different combinations of concentrations were tested (1.14 μM IAA with BA in various concentrations of 1.11–17.75 μM , or 8.88 μM BA with various IAA concentrations 0.57–9.13 μM). Excised nodal segments of axenically germinated seedlings were initially transferred to MS, supplemented with 8.88 μM BA and 1.14 μM IAA. Axillary buds started to grow on all node segments within a few days. Their stems remained short (5 to 15 mm for *G. acaulis* and *G. cruciata*, respectively) though the leaves reached a length between 25 mm (*G. acaulis*) and 120 mm (*G. cruciata*). Since only the shoots of 5–10 mm were chosen for subculturing, a four to six-fold multiplication was achieved every 4 weeks. Production of well-developed shoots was stimulated by increasing BA concentrations in the presence of 1.14 μM IAA. Indoleacetic acid concentrations higher than 2.28 μM suppressed shoot size in all investigated species. Similar observations were made by Zeleznik et al. [112] who induced shoots proliferation from *Gentiana lutea* shoot tips on MS medium supplemented with 1 mg/l of indoleacetic acid (IAA) and 0.1 mg/l benzyladenin (BA) which caused proliferation in one third of the cultured shoots in a period of 21 days.

The experiments went further in investigating the effect of more plant growth regulators. Based on the well known Murashige and Skoog nutrient medium and commonly used BAP and IAA a comparison was made with other cytokinins and auxins.

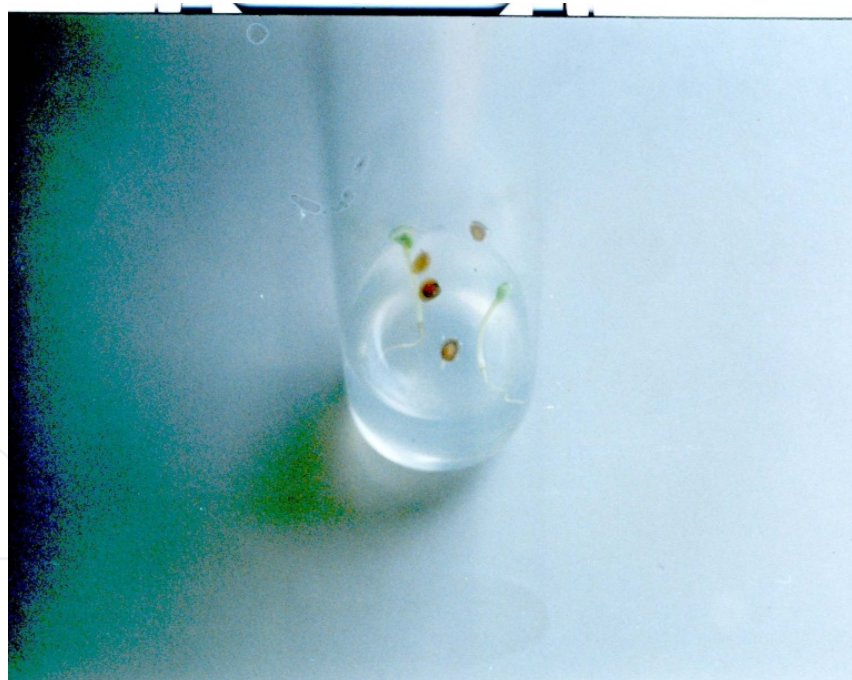


Figure 1. Seed germination of *G. lutea* on MG2 medium (MS basal medium enriched with 50 mg/l GA_3) [40].

Bach and Pawlowska [116] studied the efficacy of four cytokinins (BA, kinetin, thidiazuron, 2-iP) and gibberellin at the concentration of 1.5 μM for propagation of *Gentiana pneumonanthe*. The highest multiplication rate was achieved in the culture of the one-nodal cuttings on medium supplemented with 10.0 μM BA. In other experiments [104] media supplemented



Figure 2. *In vitro* micropropagation of *Gentiana lutea* L. on MPI medium (MS basal medium enriched with 2 mg/l zeatin and 0.2 mg/l IAA) (The mean number of shoots per explant reaching 9 in Vth passage.) [40].

with 2-iP or zeatin and IBA ensured a low multiplication of *Gentiana punctata*. Clonal propagation was slightly improved by addition of maize extract to the culture media.

Different concentrations and combinations of BAP (1 – 2 mg/l), zeatin (1 – 2 mg/l), IAA (0.1 – 0.2 mg/l), 2-iP (0.5 mg/l), and 2,4-D (0.5 mg/l) were used for bud induction and shoot multiplication of *Gentiana lutea* [40]. Best results were recorded on MP1 nutrient medium supplemented with 2 mg/l zeatin and 0.2 mg/l IAA. The mean shoot number per explant was relatively high reaching 4.57 and the average shoot height - 3.90 cm. Second in efficiency was MP3 nutrient medium supplemented with 2 mg/l BA and 0.2 mg/l IAA inducing 4.00 shoots on average per explant (Figure 2).

In vitro response may be influenced by other characteristics of the culture media like medium consistence. Sadiye Hayta et al [118] observed that efficient production of multiple shoots of *G. cruciata* L. directly from nodal segments, inducing 3.9 shoots per explants on average was

stimulated on a semi-solidified Murashige and Skoog (MS) basic medium enriched with 2.22 μM 6-benzyladenine (BA), 2.46 μM indole-3-butyric acid (IBA) [118].

In gentiana's experiments plant growth regulators were investigated not only as a factor for establishment of *in vitro* cultures but as a factor which may effect biosynthesis of the biologically active substances in the regenerated plantlets or shoots induced *in vitro*.

Similar observation about the influence of the plant growth regulators on the synthesis of secoiridoids, flavonoids and xantones was studied by Mencovic et al. [119]. There was tendency for a negative correlation between the levels of biologically active substances produced by the regenerants and the concentration of BAP and IAA added into the culture media.

Dević et al. [120] were interested in the effect of applied phytohormones on content of mangiferin in *Gentiana asclepiadea* L. *in vitro* cultures. The content of mangiferin in different plant material was determined by High Performance Liquid Chromatography (HPLC) analysis revealed that the content of mangiferin in the shoots obtained *in vitro* varied with different concentration of applied cytokinine and different auxins. There was no detectable content of mangiferin in roots obtained *in vitro* [120].

Rooting is the next crucial step after successful regeneration and multiplication of plants. Rooting was accomplished successfully in excised *Gentiana kurroo* shoots grown on MS basal medium containing 6% sucrose [107]. Pawlowska and Bach [116] observed too that *in vitro* multiplied shoots of *Gentiana pneumonanthe* formed roots on a medium without growth regulators. However, the auxins IAA, NAA, and IBA at a concentration of 0.5 μM and 1 μM stimulated rhizogenesis in excised axillary shoots with IAA demonstrating the best effect. Relatively high percentage of 52 % formation of roots from multiplied shoots of *Gentiana lutea* was achieved on MS medium supplemented with 2 mg/l of naphthalenacetic acid (NAA) [112]. Better results were reported by Petrova et al. [40] for *Gentiana lutea* when shoots were transferred to half strength MS medium enriched with either IAA (1 or 2 mg/l), IBA (2 or 3 mg/l) or NAA (0.5 or 3 mg/l). The best results of 92% and 91% rooting were obtained on half strength MS nutrient media containing 3 mg/l IBA or 3 mg/l NAA, respectively. Mean root length was almost equal in the both cases varying from 1.48 cm to 1.95 cm. Spontaneous rooting on plant *Gentiana dinarica* growth regulator-free medium occurred in some 30 % of shoot explants. Rooting was stimulated mostly by decreased mineral salt nutrition and a medium with half strength MS salts, 2% sucrose and 0.5–1.0 mg/l IBA was considered to be optimal for rooting. Wen Wei and Yang Ji [117] confirmed that the ideal medium for the rooting culture and rooting sub-culture of *G. scabra* tube seedling was 1/2 MS with 0.1 mg/l IAA and 0.3 mg/l NAA. The highest rooting of 81.7% of *G. cruciata* regenerants was also observed on half-strength MS medium supplemented with 2.46 μM IBA [118]. Beside the successful combinations of plant growth regulators inducing rooting there were reports on less favorable culture media. Butiuc-Keul et al. [104] report about failure in rhizogenesis induction in *Gentiana punctata* shoots transferred on medium supplemented with 1.0 mg/l each NAA and 2iP [104].

Acclimatization and adaptation efficiency varied with the species. In the early experiments, Pawlowska and Bach [116] achieved 65 % survival of rooted plantlets of *Gentiana pneumo-*

nanthe after being potted in soil in a greenhouse. Further, the plants were successfully planted outdoors in field conditions. These *in vitro* regenerants had a greater number of flowers and stems than plants grown in a natural habitat. *In vitro* plantlets of *Gentiana punctata* have been transferred to soil after six weeks of culture and acclimatization was successfully obtained, too [104]. Peat-based substrate for rooting plantlets of *Gentiana dinarica* was successfully used, too [115].

Turf/vermiculite mixtures were very appropriate for acclimatization of plants with well-developed roots transferred to pots in growth chambers. All the acclimatized plants (100%) survived, remained healthy and analysis of the content of secondary metabolites in the clones was determined by HPLC. The presence of gentiopicroside, loganic acid, swertiamarin, and sweroside in the samples was confirmed. Gentiopicroside was found to be the major compound [118].

For the purposes of conservation of the endangered species and for restoration of their habitats it is of a great importance to maintain the genetic stability of the regenerated plants *in vitro*. In this aspect the investigations of Kaur R et al [121] are very interesting. Genetic stability of *Gentiana kurroo* micropropagated plants maintained *in vitro* for more than 10 years was studied using randomly amplified polymorphic DNA (RAPD) and karyotype analysis. A large number of micropropagated plantlets developed from nodal segment explants were assessed for genetic variations and compared with donor mother plant maintained in the arboretum. Out of 20 RAPD primers, 5 displayed the same banding profile within all the micropropagated plants and donor mother plant. No chromosomal variations were observed by the karyotype analysis. High multiplication rate of healthy plant material associated with molecular and karyotypic stability ensures the efficacy of the protocol to be used across a long period for *in vitro* propagation of this important medicinal plant species. These results are extremely important for the application of biotechnological methods and especially of micropropagation for the multiplication of the species for their conservation when *in vitro* clones should be identical to the donor mother plants from the natural habitats.

Somatic embryogenesis is another morphogenetic pathway for regeneration of plants, which is considered the most efficient way to regenerate plants [122]. In contrast to organogenesis when the buds and shoots are not formed obligatory from one cell, a somatic embryo derives from a single cell. This way of development assures greater genetic stability and identity with the initial plant. It opened new possibilities for large-scale multiplication of valuable plants with many expectations for mass production of artificial seeds.

However, somatic embryogenesis is more difficult to obtain. Nevertheless, it was successfully induced in a number of *Gentiana* species: *Gentiana lutea* [122, 123], *Gentiana crassicaulis*, *Gentiana cruciata* [123, 124], *Gentiana pannonica* [123], *Gentiana tibetica* [123], *Gentiana pneumonanthe* and *G. kurroo* Royle [116, 123, 125, 126, 127], *Gentiana davidii* var. *formosana* (Hayata) [128], *Gentiana straminea* [103, 129].

Like in the previously described experiments for micropropagation, one of the requirements leading to success is the appropriate choice of explants. The most commonly used explants were: leaves from the first and second whorls, the apical dome, and axenic shoot culture used for *Gentiana kurroo* (Royle), *Gentiana cruciata* (L.), *Gentiana tibetica* (King. ex Hook. f.), *Gentiana*

lutea (L.), and *Gentiana pannonica* (Scop.) [123]; stem explants for initiation of callus and cell suspension cultures of *G. davidii* var. *formosana* [128]; hypocotyl (adjacent to cotyledons) of 10 days old seedlings of *Gentiana cruciata* [124]; seedling explants (root, hypocotyl and cotyledons) for *Gentiana kurroo* (Royle) embryogenic callus [125]; immature seeds (claimed to be superior initial material) of *Gentiana straminea* Maxim [129].

Plant growth regulators are the other very important factor for triggering the totipotency of the plant cell to develop somatic embryo. Unlike organogenesis and shoot formation in gentians where among the numerous tested plant growth regulators several cytokinins and auxins could be distinguished as more prominent, in the case with somatic embryogenesis it was difficult to point out the best ones. In a large number of combinations a wide spectrum of natural phytohormones and synthetic phytheregulators were examined: auxins like α -naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-o-anisic acid (dicamba), and cytokinins: zeatin, 6-furfurylamonopurine (kinetin), N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (TDZ), N-(2-chloro-4-pyridyl)N'-phenylurea, 6-benzylaminopurine (BAP) or benzyladenine (BA), and adenine sulfate. However, the natural auxin indoleacetic acid is not seen in this list. It makes impression that more auxins of synthetic origin are involved in the studies.

The role of the plant growth regulators will be illustrated by several examples of establishment of cell suspension cultures and somatic embryogenesis. One of the pioneer investigations was performed by Fu-Shin Chuen et al. [128]. Fast-growing suspension cell cultures of *G. davidii* var. *formosana* were established by subculturing callus, which was initiated from stem explants on MS basal medium supplemented with 0.2 mg/l kinetin and 1.0 mg/l NAA. Cell suspension growth was maintained in liquid MS basal medium supplemented with 0.2 mg/l kinetin and 3% sucrose. The cultures were incubated on an orbital shaker (80-100 rev/min) at $25 \pm 1^\circ\text{C}$ and low light intensity ($2.33 \mu\text{Em}^{-2}\text{s}^{-1}$). The low pH of 4.2-5.2 was crucial for the successful cell division and growth.

Quite interesting work from the early period of somatic embryogenesis was that one of Mikula et al., [124]. Authors investigated the effect of phytheregulators on *Gentiana cruciata* structure and ultrastructure changes occurring during tissue culture. MS induction medium containing 1.0 mg/l dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80 mg/l adenine sulfate was used for culturing of hypocotyl (adjacent to cotyledons) explants from 10 days old seedlings. During the first 2 days of culture cell division of epidermis and primary cortex was the first response. Numerous disturbances of karyo- and cytokinesis were observed, leading to formation of multinuclear cells. With time, the divisions ceased, and cortex cells underwent strong expansion, vacuolization and degradation. About the 6th day of culture, callus tissue was formed and the initial normal divisions of vascular cylinder cells were observed. Cells originating from that tissue were small, weakly vacuolated, with dense cytoplasm containing active-looking cell organelles and actively dividing leading to formation of embryogenic callus tissue. During the 6-8th week of culture, in the proximal end of the explant, masses of somatic embryos were formed from outer parts of intensively proliferating tissue. Production of somatic embryos was more effective from suspension culture than from agar medium. Liquid culture made it possible to maintain the cell suspension's embryogenic competence for 5 years.

Quite vast and extensive studies on the establishment of gentians embryogenic cultures and their biotechnological potentials were carried by a research group with impressive publishing activity [114, 126, 130, 131]. Culture initiation and intensive callus proliferation of *Gentiana cruciata* were stimulated by 2,4-D and kinetin using various explants [130]. However, only some of the tissues of initial explant were able to form embryogenic callus. Cytological, ultrastructural and scanning analysis brought evidences that almost each of the cotyledon cells responded by callus formation and somatic embryo differentiation. Central cylinder of the hypocotyls gave the best response for embryogenic proliferation compared to other tissues of hypocotyls. Another medium containing 1.0 mg/l dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80 mg/l SA proved to be very efficient to maintain very long-term cell suspension cultures of proembryogenic masses. Long-term culture provided opportunities for numerous analysis to have evidences of the single cell origin of somatic embryos which originated from freely suspend single cells or single cells from the embryogenic clusters. Medium supplemented with GA₃ helped to complete development and stimulated the somatic embryo conversion in germlings. Embryogenic potential was genotype dependent with *G. tibetica* and *G. kurroo* being outstanding generating more than hundreds somatic embryos from 100 mg of tissue for more than two years. Interestingly the regeneration ability was maintained not only in the long-term suspension cultures but it was demonstrated in the protoplast cultures, too [126].

Protoplasts with very high viability ranging from 88 to 96 % were isolated from cell suspensions derived from cotyledon and hypocotyl of *Gentiana kurroo* [126]. Three techniques of culture and six media were evaluated in terms of their efficiency in producing viable cultures and regenerating entire plants. The best results of plating efficiency (68.7% and 58.1% for cotyledon and hypocotyl derived suspensions, respectively) were obtained with agarose bead cultures in medium containing 0.5 mg/l 2,4-D and 1.0 mg/l kinetin. Regeneration of plants was also possible when embryos were transferred to half-strength MS medium. However, flow cytometry analysis revealed increased amounts of DNA in about one third of the regenerants which limits the application of isolated protoplasts in the programs for conservation and reproduction of an endangered species. Hence, the efforts were directed again to cell and tissue cultures examining the factors for efficient and reliable plant regeneration, even to examining photosynthetic activity in dependence of the sucrose content in the embryogenic culture media [126].

Fiuk and Rybczyn'ski [123, 125] expanded their studies using leaves derived from axenic shoot culture of five *Gentiana* species (*Gentiana kurroo*, *Gentiana cruciata*, *Gentiana tibetica*, *Gentiana lutea*, and *Gentiana pannonica*) and cultured on MS basal medium supplemented with three different auxins: 2,4-D, NAA, or dicamba in three concentrations of 0.5, 1.0, or 2.0 mg/l; and five different cytokinins: zeatin, kinetin, BAP, TDZ, and N-(2-chloro-4-pyridyl)N'-phenylurea in concentrations between 0.25 and 3.0 mg/l depending on the cytokinin activity. After two months the percentage of embryogenesis was the highest for *G. kurroo* reaching 54.7% and depending on plant growth regulators. This gentian was the only species responding to the all tested combinations of auxins and cytokinins, while none of the 189 induction media stimulated somatic embryogenesis from *G. lutea* explants. Efficiency of embryogenesis was genotype dependent *G. tibetica* and *G. cruciata* both produced an average of 6.6 somatic

embryos per explant, while *G. pannonica* and *G. kurroo* regenerated at 15.7 and 14.2 somatic embryos per explant, respectively. Optimum regeneration was achieved in the presence of NAA combined with BAP or TDZ. NAA also stimulated abundant rhizogenesis. Somatic embryos were also regenerated from adventitious roots of *G. kurroo*, *G. cruciata*, and *G. pannonica*. Somatic embryos developed easily into plantlets on half strength MS medium.

The same research group extended its investigations on the factors influencing efficiency of somatic embryogenesis in cell suspension of *Gentiana kurroo* (Royle) - the species revealing the best morphogenic potential in their previous studies [125]. Suspension cultures were initiated in liquid MS medium supplemented with 0.5 mg/l 2,4-D and 1.0 mg/l kinetin from embryogenic callus derived from seedling roots, hypocotyls and cotyledons. Unexpectedly the highest growth rate was observed for root derived cell suspensions. Further more differences in aggregate structure depending on their size were detected by microscopic analysis. In order to assess the embryogenic capability of the particular culture, 100 mg of cell aggregates were implanted on MS agar medium supplemented with 0–2 mg/l kinetin, 0–2 mg/l GA₃ and 80 mg/l adenine sulfate. The highest number of somatic embryos was obtained for cotyledon-derived cell suspension on GA₃-free medium, but the presence of the other plant growth regulators (0.5–1.0 mg/l kinetin, 0.5 mg/l GA₃ and 80 mg/l adenine sulfate) determined the best morphological quality of embryos. The morphogenic competence of cultures also depended on the size of the aggregate fraction and was lower when size of aggregates decreased. Flow cytometry analysis revealed 100% uniformity for regenerants derived from cotyledon suspension but lack of uniformity of plantlets obtained from hypocotyls suspension. These observations were of great significance for the choice of appropriate explants and culture media conditions for the multiplication of a particular gentian species via somatic embryogenesis.

Cai YunFei et al. [129] confirmed the role of the explant and its interaction with the plant growth regulators added into the media of *Gentiana straminea* Maxim. They observed that calli induced from immature seeds were superior to those from hypocotyls or young leaves in regeneration via somatic embryogenesis and demonstrated that 2,4-D was efficient for both callus induction and embryogenesis, IAA is suitable for embryogenic callus proliferation, and BAP promotes both embryo development and the accumulation of gentiopicroside in the cultures. Experiments went further in exploring *Gentiana in vitro* cultures potentials by selecting regenerated plants for high gentiopicroside content. A highly productive clone was selected. Its cells contained 5.82 % of gentiopicroside, which levels were two folds higher than the control plants (1.20–3.73 %). Genetic stability of the regenerated plants was also proved both by cytological and random amplified polymorphic DNA analyses.

Similar experiments were performed with *Gentiana straminea* Maxim. MS medium supplemented with 2 mg/l 2,4-D and 0.5 mg/l BA was the best medium for embryogenic callus induction from leaf explants [103]. Genetic stability of the regenerants was assessed by 25 inter simple sequence repeat (ISSR) markers. Out of 25 ISSR markers, 14 produced clear, reproducible bands with a mean of 6.9 bands per marker confirming that the regenerants maintained high genetic fidelity.

One of the recent reports [122] presented interesting results for the possibility to use recurrent somatic embryogenesis in long-term cultures of *Gentiana lutea* for production of synthetic

seeds. After induction of somatic embryogenesis in the presence of auxins in the first cycle of *in vitro* cultures, recurrent somatic embryogenesis was performed in long-term cultures in the absence of phytohormones but in the presence of the sugar alcohols mannitol and sorbitol. Adventive somatic embryos were generated continuously at a high rate along with maturation, germination and development into plants.

One of the possibilities of biotechnology for conservation of rare species is the establishment of *in vitro* germplasm banks, which may include cryopreservation of *in vitro* multiplied valuable plant material. There are several interesting publications of one research group dedicated to this problem [124].

For preservation of proembryogenic masses of *G. cruciata*, four protocols of cryopreservation were studied: direct cooling, sorbitol/DMSO treatment, vitrification, and encapsulation. Direct cooling and sorbitol/DMSO treatment was unsuccessful. Vitrified tissue required a minimum 3 weeks culture on solid medium for cell proliferation to reach the proper fresh weight for manipulation. Alginate beads with PEMs were transferred directly to liquid medium for post-freezing culture. Vitrification and encapsulation maintained high viability of post-freezing proembryogenic masses, but encapsulation ensured faster restoration of *G. cruciata* cell suspension [124]. A reliable technique for cryopreservation by encapsulation was developed for two suspension cultures of *Gentiana* species (*Gentiana tibetica* and *G. cruciata*) of different ages and embryogenic potential. A water content of 24-30% (fresh weight basis) after 5-6 h dehydration of encapsulated cells of gentians yielded the highest survival (68% for *G. tibetica* and 83% for *G. cruciata*) after cryopreservation. Flow cytometry showed that cryopreservation did not change the genome size neither of the somatic embryos nor of the regenerants [132]. The embryogenic cell suspension culture of *Gentiana cruciata*, cryopreserved by the encapsulation/dehydration method, survived both short- (48 h) and long-term (1.5 years) cryostorage with more than 80% viability. The (epi)genetic stability of 288 regenerants derived from: non-cryotreated, short-term, and long-term cryo-stored tissue was studied using metAFLP markers and ten primer combinations. AFLP alterations were observed but they were not associated with the use of cryopreservation, but were probably related to the *in vitro* culture processes [133]. These results gave great hopes for the use of cryo-techniques in preservation of valuable medicinal species.

Genetic transformation was also applied to gentiana species aiming at obtaining higher production of biologically active substances or biosynthesis of new valuable compounds. *Agrobacterium rhizogenes* mediated transformation was achieved in shoots of micropropagated *Gentiana acaulis*, *G. cruciata*, *G. lutea*, and *G. purpurea* inoculated with suspensions of *Agrobacterium rhizogenes* cells [134, 135]. Few years later Menkovic et al [119] after infection with *Agrobacterium rhizogenes* managed to obtain nine hairy root clones which differed in the amount of secondary metabolites. *Agrobacterium tumefaciens* was also used for inoculation of *Gentiana punctata* [136] and *Gentiana dahurica* Fisch by *A. tumefaciens* [137]. However, due to the great opposition in many countries against the genetically modified organisms, especially these ones with potential use in food and nutraceutical industries genetic transformation experiments remained more in the laboratory mainly to study the metabolic pathways.

Genus *Leucojum*. *Leucojum aestivum* (summer snowflake) is one of the most worshiped medicinal plants on the Balkan region and in the world. *Leucojum aestivum* L. (Amaryllidaceae family) is a polycarpic geophyte distributed in the wetlands of Central and South Europe (Mediterranean and the Balkans) and in West Asia. *L. aestivum* grows on alluvial soils with high nitrogen levels. The mean size of the plants increased with the water content of the soil. Seed reproduction is whimsical. Seed set of the plants was not influenced by the size of a population, but strongly increased with the density of flowering plants. Optimal temperature for seed germination is 20-25°C [138]. Overharvesting of its bulbs for medical purposes has brought to a destruction or alteration of its habitats across Europe [138]. Therefore, summer snowflake has turned into an endangered species and is protected in several European countries (e.g. Bulgaria, Hungary and Ukraine).

Leucojum aestivum L. is used as a source of galanthamine - an isoquinoline alkaloid produced exclusively by plants of the family Amaryllidaceae (mainly belonging to the genus *Galanthus*, *Leucojum* and *Narcissus*). Due to its acetylcholinesterase inhibitory activity, galanthamine is used for various medical preparations for the treatment of neurological disorders and especially for senile dementia (Alzheimer's disease) and infantile paralysis (poliomyelitis). A very effective Bulgarian remedy to cure poliomyelitis was produced from *L. aestivum* in the middle of the XXth century. This marked tremendous interest and respect of the plant and enormous demands for raw material. Despite the possibility for organic synthesis, galanthamine is still extracted from natural sources. For industrial purposes *L. aestivum* plants are harvested from wild populations in their natural habitats which causes increasing problems regarding quality of the plant material as well as natural populations depletion. The limited availability of the plants and the increasing demands for this valuable metabolite has imposed urgent search for alternative approaches both for protection of the species and for production of galanthamine. In this respect biotechnology methods could be used for *in vitro* storage of genotype accessions from different natural populations with proven alkaloid profiles, for rapid propagation of this threatened medicinal plant for both industry and natural resource protection, and for production of its valuable biologically active substances under controlled conditions. However, not so much data are available in the literature concerning *in vitro* cultures of this plant.

The Bulgarian scientists Stanilova et al. [38] and Zagorska et al [139] are pioneer in establishment of *in vitro* cultures and micropropagation of *Leucojum aestivum*. One of the prerequisites for their success was the elaboration of a successful procedure for decontamination of the plant material gathered from nature. Plant material should be used 42 days after collecting. The bulbs were rinsed for 16 hours with stream water followed by immersion in 70% ethanol for 30 s and sterilized with 0.1% HgCl₂ for 3 min. Relatively good decontamination was achieved for leaf explants applying hypochlorites – 47.46% using 5% Ca (ClO)₂ for 6 min and 54.76% - 15% NaClO for 20 min. During their initial studies of the morphogenetic potential of the basal and apical parts of bulbs, stems, leaves and ovaries it was observed that the scales of *L. aestivum* possessed the highest regenerative ability producing 4.08 - 4.19 regenerants per explant. Whereas leaf explants had lower regeneration potential – 1.67 regenerants per explant. Murashige and Skoog (MS) medium supplemented with 1 mg/l benzyladenine (BA) and 1 mg/

l kinetin as well as Linsmaier and Skoog (LS) medium enriched with 0.5 mg/l NAA and 0.1 mg/l kinetin proved to be the most suitable for direct organogenesis [38]. Rhizogenesis was induced on MS basal medium with reduced sugar content of 15 g/l and enriched with 0.1 mg/l NAA, 0.1 mg/l kinetin and 0.1 mg/l BAP. Further investigations focused on *in vitro* clonal propagation of *L. aestivum*. Twenty four clones were obtained and most of them demonstrated high regeneration rates and stable alkaloid profiles. Galanthamine levels of some of the *in vitro* obtained clones was as high as galantamine levels of commercially important representative of Bulgarian *L. aestivum* populations. Five clones: four galanthamine-type and one lycorine-type were selected as promising for further investigations [140].

In Turkey Karaoglu [141] confirmed the effectiveness of bulb-scales explants for micropropagation of *Leucojum aestivum* and tested immature embryos for initiation of *in vitro* cultures. Using 2 and 4 bulb-scales explants the highest number of bulblets (6.67 and 5.83) were achieved on MS medium containing 1 mg/l BA and 1 mg/l NAA or 2 mg/l BAP and 0.5 mg/l NAA, respectively. Regeneration capacity of immature embryos was twice lower reaching 2.27 bulblets on MS medium containing 0.5 mg/l BA and 4 mg/l NAA. The best rooting of bulblets regenerated from bulb scales was obtained on MS medium containing 1 mg/l NAA. Rooted bulbs were finally transferred to compost and acclimatized to ambient conditions [141].

Later *in vitro* cultures of *Leucojum aestivum* were reported in Hungary. Kohut et al. [142] succeeded to obtain from 81 % to 92 % contamination free material. Prior to surface sterilization the old leaves and roots were dissected from the bulbs and they were stored at low temperature of 2–3°C for 1 and 5 week periods. The bulbs, bulb scales and leaves of the bulbs were placed on MS medium containing 1 mg/l BA and 0.1 mg/l NAA.

Shoot *in vitro* cultures were initiated also from bulb explants in others' experiments [143]. However, Gamborg's B5 medium was used for the initiation and maintaining of the cultures, which were kept in darkness. This medium contained 30 g/l of sucrose, 1 mg/l 2,4-D, 0.5 g/l casein hydrolysate, 2 mg/l adenine, and 10 mg/l glutathione. The *in vitro* cultures were subcultured at 2.5 month intervals in MS medium supplemented with 1 g/l Ca(NO₃)₂, 0.5 mg/l BAP, 0.01 mg/l IBA, and 2.93 mg/l paclobutrazol. During the subcultures, shoot-clumps which were formed were cut to increase the number of explants, and the newly formed shoot clumps were separated. The *in vitro* cultures were maintained at 23-25° C with a 16/8 h light /dark photoperiod. Later the same research group [144] offered a three step protocol for *in vitro* long-term conservation of *L. aestivum* which was used to create a genebank with accessions from 31 Bulgarian populations. For *in vitro* cultures dormant bulbs were used, which were cut into 8, 16 or more segments. For sterilization, these segments called "twin-scale" were treated with 70% ethanol for 30 s and sterilized with 1% HgCl₂ for 3 min. The development of the shoot-clumps started from the basal parts of the scales at the end of the first week. The development of *in vitro* shoot-clump cultures was tested on three nutrient media: MS, B5, and QL with or without plant growth regulators, BAP (0.5 - 3.0 mg/l), IBA (0.01 - 1 mg/l) NAA (0.2 - 2 mg/l) and TDZ (1 - 2 mg/l), sucrose (0 - 120 g/l), and charcoal (2g/l). Shoot-clumps were obtained, from explants cultivated on B5 medium (6), supplemented with 0.5 g casein hydrolyzate, 1 mg/l 2,4-D, 10 mg/l adenine, 10 mg/l glutathione, 30 g/l sucrose, 6 g/l agar. The fastest multiplication however was observed on MS medium with 30 g/l sucrose, 2 mg/l BAP, 1.15 mg/l

NAA. Increasing sucrose concentration up to 90 g/l resulted in higher mass of the obtained bulbs. About 1000 regenerated bulbs with well-developed roots were successfully adapted at *ex vitro* conditions. Authors observed that plant *ex vitro* adaptation depended on the bulb size. The biggest bulbs (over 1.5 cm in size) were the most adapted (99 %) whereas about 60% of the medium size bulbs (0.5-1.5 cm) and 20% of the small bulbs (less than 0.5 cm) survived. Mainly easily rooting bulbs were formed on hormone free nutrient medium (MS with vitamins, sucrose-30 g/l, charcoal - 2 g/l, and pH-5.6) [144].

Callus cultures from young fruits of *Leucojum aestivum* L. were obtained, too [145]. Non-differentiated cell growth was stimulated by high concentrations of the auxin 2,4-D (4 mg/l) and the cytokinin BA 2 mg/l. Callus tissue formed regenerants when 1.15 mg/l NAA and 2 mg/l BA were added to the MS medium.

Somatic embryos were formed from callus tissues cultivated on MS medium containing 2 μ M or 5 μ M picloram (4-amino- 3,5,6-trichloropicolinic acid) and 0.5 μ M BAP [146]. Regeneration of plants was possible on medium enriched with zeatin (0.5 μ M). Authors observed that the processes of differentiated or non-differentiated growth leading to somatic embryogenesis or callus growth, respectively, were influenced by ethylene or its precursor ACC (1-aminocyclopropane-1-carboxylic acid). At higher concentrations (25 μ M) of picloram callus cultures produced ethylene (9.5 nL/g fresh weight: F.W.) whereas no ethylene was detected in cultures of somatic embryos cultivated on medium supplemented with 0.5 μ M NAA and 5 μ M zeatin. Application of ACC increased ethylene production thus suppressing callus growth and enhancing somatic embryos induction and globular embryos development. Another effect of ACC was to induce galanthamine production in somatic embryo cultures (2% dry weight). However, galanthamine production in callus cultures was induced by silver thiosulphate (STS) though in low levels (0.1% dry weight). These results are promising for use of somatic embryos cultures in bioreactors for production of galanthamine [146].

Alkaloid content in *Leucojum aestivum* wild plants and their *in vitro* cultures was studied in a series of experiments carried out by a Bulgarian research group [143, 145, 147 - 150]. Callus cultures were obtained from young fruits of *Leucojum aestivum* on MS nutrient medium supplemented with 4 mg/l 2,4 D and 2 mg/l BAP. Further, shoot cultures were established by subculturing the obtained calli on the same nutrient medium supplemented with 1.15 mg/l NAA and 2.0 mg/l BAP. These *in vitro* systems were used to study the growth and galanthamine accumulation. The authors observed that the amount of accumulated galanthamine strongly depended on the level of tissues differentiation. The maximum yield of biomass (17.8 g/l) and the maximum amount of accumulated galanthamine (2.5 mg/l) were achieved under illumination after the 35th day of submerged cultivation of one of the lines *L. aestivum* -80 shoot culture.

The alkaloids of intact plants, calli and shoot-clump cultures of *L. aestivum* were analyzed by capillary gas chromatography – mass spectrometry (CGC-MS). In one series of experiments fourteen alkaloids of galanthamine, lycorine and crinine types were identified (11 in the intact plants and eight in the *in vitro* cultures) in alkaloid mixtures extracted from intact plants and *in vitro* cultures. Excellent peak resolution for the alkaloids was exhibited and isomers of galanthamine and N-formylnorgalanthamine were well separat-

ed [147]. Applying the same methods of CGC-MS, extracts from bulbs collected from 18 Bulgarian populations and from shoot-clumps obtained *in vitro* from eight different populations were subjected to analysis and nineteen alkaloids were detected. Typically, galanthamine type compounds dominated in the alkaloid fractions of *L. aestivum* bulbs but lycorine, haemanthamine and homolycorine type alkaloids were also found as dominant compounds in some of the samples. Galanthamine or lycorine as main alkaloids presented in the extracts from the shoot-clumps obtained *in vitro*. The galanthamine content ranged from traces to 454 µg/g dry weights in the shoot-clumps while it was from 28 to 2104 µg/g dry weight in the bulbs [143]. In other investigations twenty-four alkaloids were detected analyzing intact plants, calli and shoot-clump cultures. Shoot-clumps had similar profiles to those of the intact plant while calli were characterized with sparse alkaloid profiles. Seven shoot-clump clones produced galanthamine predominantly whereas another three were dominated by lycorine. It was also observed that illumination stimulated accumulation of galanthamine (an average of 74 µg/g of dry weight) in shoot-clump strains while in darkness galanthamine levels were two folds less (an average of 39 µg/g of dry weight). The shoot-clumps, compared to intact plants, accumulated 5-folds less galanthamine. The high variability of both the galanthamine content (67% and 75% of coefficient of variation under light and darkness conditions, respectively) and alkaloid patterns indicated that the shoot-clump cultures initiated from callus could be used as a tool for improvement of the *in vitro* cultures production of the valuable substances [148]. The investigations extended on the alkaloid patterns in *L. aestivum* shoot culture cultivated at temporary immersion conditions where 18 alkaloids were identified, too. The temperature of cultivation influenced enzyme activities, catalyzing phenol oxidative coupling of 4'-O-methylnorbelladine and formation of the different groups *Amaryllidaceae* alkaloids. Decreasing the temperature of cultivation of *L. aestivum* 80 shoot culture led to activation of para-ortho' phenol oxidative coupling (formation of galanthamine type alkaloids) and inhibited ortho-para' and para-para' phenol oxidative coupling (formation of lycorine and haemanthamine types alkaloids). The *L. aestivum* 80 shoot culture, cultivated at temporary immersion conditions, was considered a prospective biological matrix for obtaining wide range of *Amaryllidaceae* alkaloids, showing valuable biological and pharmacological activities [150]. The most recent report was about successful cultivation of shoot culture of summer snowflake in an advanced modified glass-column bioreactor with internal sections for production of *Amaryllidaceae* alkaloids. The highest amounts of dry biomass (20.8 g/l) and galanthamine (1.7 mg/l) were obtained when shoots were cultured at temperature of 22°C and 18 l/(l h) flow rate of inlet air. At these conditions, the *L. aestivum* shoot culture possessed mixotrophic-type nutrition, synthesizing the highest amounts of chlorophyll (0.24 mg/g DW (dry weight) chlorophyll A and 0.13 mg/g DW chlorophyll B). The alkaloids extract of shoot biomass showed high acetylcholinesterase inhibitory activity ($IC_{50} = 4.6$ mg). The gas chromatography-mass spectrometry (GC/MS) profiling of biosynthesized alkaloids revealed that galanthamine and related compounds were presented in higher extracellular proportions while lycorine and hemanthamine-type compounds had higher intracellular proportions. The developed modified bubble-column bioreactor with internal sections provided conditions ensuring the growth and galanthamine production by *L. aestivum* shoot culture

[149]. The influence of the nutrient medium, weight of inoculum, and size of bioreactor on both growth and galanthamine production was studied in different bioreactor systems (shaking and nonshaking batch culture, temporary immersion system, bubble bioreactor, continuous and discontinuous gassing bioreactor) under different culture conditions. The maximal yield of galanthamine (19.416 mg) was achieved by cultivating the *L. aestivum* shoots (10 g of fresh inoculum) in a temporary immersion system in a 1l bioreactor vessel which was used as an airlift culture vessel, gassing 12 times per day (5 min) [151].

Completely different types of experiments were the attempts of genetic transformation with *Agrobacterium*. *Agrobacterium rhizogenes* strain LBA 9402 has been tested [152] for its capacity to induce hairy roots of this monocotyledonae plant. Diop et al. [152] have developed an efficient transformation system for *L. aestivum*, which could be used to introduce genes encoding enzymes of isoquinoline alkaloid biosynthesis into *L. aestivum* to enhance the production of target molecules in this medicinal plant. However, the transformed roots obtained did not synthesize galanthamine.

At this stage of *in vitro* research establishment of organogenic cultures and optimization of galanthamine production by differentiated cells using the methods of biotransformation are more promising and reliable.

Genus *Rhodiola* is highly varied among others in family *Crassulaceae* (comprising of 1500 species in 35 genus). The genus *Rhodiola* includes over 200 quite polymorphic species, out of which 20 species (*Rh. alternata*, *Rh. brevipetiolata*, *Rh. crenulata*, *Rh. kirilowii*, *Rh. quadrifida*, *Rh. sachalinensis*, *Rh. Sacra* etc.) have pharmacological properties and are used for production of medical preparations [153].

Intensive and unscrupulous exploitation of the natural habitats in many countries has led to extinction of these species in these regions [154]. This provoked nature-protecting measures to be undertaken like (1) cultivation under appropriate conditions, (2) protection of the populations in the protected areas, (3) including the species in Red Books of rare and endangered plants species. *Rhodiola* species contain various quantities of salidroside – one of the most important ingredients in the biological active complex [155 - 159]. Salidroside content in plants varies depending on the genetical structure, the developmental stages, the plant age, the ecological and agrobiological conditions [160] what is one of the reasons for the scientists to look for conditions minimizing these effects by biotechnological way of more controlled production of this biologically active compound. From another hand extracts from medicinal plants are rich in other metabolites bringing to the multiple health benefits [159] what stimulates search and identification of more biologically active substances which can be produced in cultures *in vitro*.

In Bulgaria *Rhodiola rosea* (Golden root, Roose root) (*Sedum roseum* (L.) Scop., *S. rhodiola* DC.) is under protection of the Act for biological diversity [161]. *Rhodiola rosea* is included in the Red Books of Republic of Buryatia AR, of Yakut ASSR, of Mongolia; "Rare and Extinct Plant Species in Tyva Republic," "Rare and Extinct Plant Species in Siberia," in Great Britain—Cheffings & Farrell, in Finland—category "last concerned." (according to IUCN Red List Categories and Criteria: Version 3.1 (IUCN, 2001).

Rhodiola rosea species are worshiped for their roots and rhizomes therapeutical role in many diseases. *Rhadi et Rhizoma Rhodiolae* of *Rh. rosea* are used in medicine for optimization of own-body biochemical and functional reserves of the organism, for stimulation of body's nonspecific resistance for regulation of the metabolism, central nervous system, cardiovascular system and the hormonal system [162 – 164] for rehabilitation after heavy diseases, for prophylactics of onco disease [153, 159, 165], etc. *Rhodiola quadrifida* (Pall.) Fisch. et May is a perennial grassy plant growing predominantly in some highland regions of the former USSR (Altai, Sayan), in East Siberia, in some mountainous regions of China (Sichuan) and in high mountain regions of Mongolia. It is used in traditional medicine of Mongolia and Tibet, against fatigue, stress, infections, inflammatory diseases and protection of people against cardiopulmonary function problems when moving to high altitude [166; 167]. The phytochemical composition of the ingredients (without cinnamic alcohol and rosiridin) is similar to that of *Rh. rosea* [168]. *Rhodiola kirilowii* is a Chinese medicinal herb. Roots and rhizomes extracts are used in Asiatic medicine independently of their adaptogenic properties also as antimicrobial and anti-inflammatory drugs [169, 170]. *Rhodiola sacra* grow in the Changbai Mountain area, Tibet and Xinjiang autonomous regions in China. In Tibetan folk medicine, *Rhodiola Radix* is used as a hemostatic, tonic and contusion releaf factor. Positive effects on learning and memory have been reported, too [171, 172]. *Rhodiola crenulata* is distributed in the high cold region of the Northern Hemisphere in the high plateau region of southwestern China, especially the Hengduan Mountains region including eastern Tibet, northern Yunnan and western Sichuan. It has strong activities of anti-anoxia, antifatigue, anti-toxic, anti-radiation, anti-tumour, anti-aging, and active-oxygen scavenging [173, 174]. *Rhodiola sachalinesis* A. Bor. is used as a drug of “source of adaptation to environment” in Chinese traditional medicine. Salidroside can effectively enhance the body's ability to resist anoxia, microwave radiation, and fatigue. Furthermore, its effect on extending human life was also found [175]. *Rhodiola imbricata* Edgew commonly known as rose root, is found in the high altitude regions (more than 4000 m altitude) of India. The radioprotective effect, along with its relevant superoxide ion scavenging, metal chelation, antioxidant, anti-lipid peroxidation and anti-hemolytic activities were evaluated under both *in vitro* and *in vivo* conditions [176]. *Rhodiola iremelica* Boriss. – is an endemic plant of Middle and South Ural mountain. It is included in the Red Book of Republic of Bashkortostan (Bashkiria) in the category of rare and endangered species. *Rh. iremelica* is located in places with different climatic conditions making them unique [177].

Despite the incontestable/undisputed interest to *Rh. rosea* and the wide intensive research in phytochemistry, the potential area of the plant biotechnologies, remains less studied and exploited in comparison to other medicinal species. Some of the researchers studied the possibility for induction of calli cultures, biotransformation and organogenesis. Other authors focus their research on *Rhodiola* potential for regeneration and investigation of biologically active substances. Experiments are focused mainly in two directions: 1) looking for possibilities for *in vitro* synthesis of valuable metabolites and/or 2) establishment of effective systems for micropropagation, for reintroduction of the plant in nature or in the field for protection of the species.

Pioneer experiments on golden root *in vitro* cultures were initiated 20 years ago [178] from a Russian scientist who described rooting of assimilating of sprouts *R. rosea*. Later a few other reports have appeared concerning the effect of culture media composition and of explant type on the ability for callogenesis, organogenesis and regeneration of *R. rosea*, as well as other factors influencing growth and morphogenesis. Using leaf segments Kirichenko et al. [179] studied callus and regeneration ability for propagation *in vitro* of rose root while Bazuk et al. [180] focused on the rooting potential of shoots obtained from stem segments with two adjacent leaves. Investigations that are more detailed were carried using seeds and rhizomes from three ecotypes from the High Altai and South Ural region, which served as the explant source to study induction of callogenesis and organogenesis [181]. Explant development was observed on MS media containing various phytohormones (BAP, IAA, NAA, IBA, 2,4-D). Very high percentage of 86% of the explants formed abundant calli on MS medium supplemented with 0.1–0.2 mg/l IAA. BAP and IAA in concentrations of 0.2 mg/l and 0.1 mg/l, respectively, was the optimal combination for multiple bud formation in *Rhodiola rosea* from stem segments, while for *Rhodiola iremelica* the efficient concentrations were lower—0.1 mg/l BAP and 0.05 mg/l IAA. The processes of efficient callogenesis and organogenesis were influenced by ecotype differences. Adaptation of regenerants in vermiculite for two weeks in conditions of high humidity (85–90%) and later in mixture of soil, peat, and vermiculite in proportion of 1 : 1 : 1 was successful, but with considerable differences in the survival rate (from 10% to 95%). In the later experiments [182], the effect of 5% or 10% v/v liquid extracts of *Rh. rosea* extracts on the morphogenic abilities of *Rh. rosea* and *Rh. iremelica* were studied. Different *in vitro* responses were provoked. Bud induction was stimulated by the lower concentration and inhibited by the higher ones leading to formation of 8.5 shoots per explant in the first case and 1.1 in the second case.

Unlike the previously described investigations with the Altai ecotype of *Rhodiola rosea* the optimal concentrations of the cytokinin BAP were 10–15-fold higher for induction of *in vitro* cultures from immature leaves explants from a Tibetan ecotype of golden root [183]. The authors noted interaction between the growth regulators and the illumination of the cultures. Two mg/l BA and 0.2 mg/l NAA added to the MS medium stimulated formation of incompact callus tissue. However, when explants were cultivated under dark conditions, higher concentrations of the same phytohormones BA (3 mg/l) and NAA (0.25 mg/l) were more efficient. MS medium containing 2 mg/l BA and 0.25 mg/l NAA induced shoot multiplication while rooting was induced on MS medium containing 0.5 mg/l or 1 mg/l IAA.

In Bulgaria the first investigations on *Rhodiola rosea* were on the content of polyphenols and salidroside in the local populations in Rila, Pirin and Balkan Mountain [184]. The highest salidroside levels in the rhizome and root were found in the plants from Rila Mountain while the lowest ones in the plants from Pirin Mountain 1.55 % and 0.72 %, respectively. From another side polyphenols were in highest concentration in rose root from Pirin population. Salidroside is accumulated in roots and rhizomes while polyphenol content is equal in all parts of the plant [185, 186].

Seeds of *Rhodiola rosea* lose their germination potential for a relatively short period of time compared to other species. Stratification is one of the approaches for overcoming this problem. Revina et al. [187] reported about higher germination up to 75 % after treatment of seeds for one month at temperatures of 2–4 °C. Other authors confirm the role of stratification [188] and report

about stimulation of germination up to 50-75 % after subjecting seeds to lower temperatures of -5°C for a period of 3 months [189]. Dimitrov et al. [190] applied a new approach for *in vitro* cultivation of seeds. Golden root germination of seeds started on the 7th day of cultivation and lasted until the 40th day reaching from 37.5% to 97.0% depending on media composition. Germination was stimulated when seeds were cultured on MS basal medium enriched with 50-100mg/l gibberellic acid. These high concentrations of GA₃ enhanced germination while lower concentrations of 5-25 mg/l GA₃ favored obtaining of seedlings with bigger size [43].

The initial investigations for establishment of golden root *in vitro* cultures in Bulgaria were dedicated to find out a suitable ecotype for *in vitro* experiments [190 – 192]. Tasheva et al. [193, 194] optimized seed germination *in vitro* and later report the first successful results for *in vitro* propagation of *Rhodiola rosea*. A large number of explants isolated from *in vitro* seedlings (stem segment with leaf node, apical bud, explants excised from the seedling root basal area) and *in vivo* plant (apical bud, adventitious shoots, stem explant, rhizome buds, rhizome segments) were used to study *in vitro* response [195] on Murashige and Skoog (MS) basal medium containing various hormonal combinations including benzyladenine, kinetin, zeatin, 2-ip etc. *In vitro* development led to formation of plantlets, leaf rosette, various type of callus (compact green, pale, soft liquidy) and callus degeneration without bud formation. The authors observed that the explants of seedling and apical bud are more suitable for mass clonal propagation. Multiple shoots proliferation from leaf node explants was most effective on nutrient medium containing 1.0 or 2.0 mg/l zeatin, 0.1 mg/l IAA and 0.4 mg/l GA₃ (Figure 3 a, Figure 3 b). Rooting *in vitro* proved to be the most efficient on nutrient medium containing IBA (2.0 mg/l), IAA (0.2 mg/l) and GA₃ (0.4 mg/l) [195]. Interestingly, it was observed that the coefficient of propagation varied during the different seasons. Highest level of proliferation was recorded in May-June, when the mean number of shoots per explant was 6.78, while during the cold seasons multiplication was relatively lower with 2.11 shoots per explant [196]. Adaptation of obtained plants was done under controlled conditions in a cultivation room for 2-3 months and later grown plants were transferred to green house, where survival rate reached high levels of 85% (Figure 3 c). After 6 months, these regenerants were rooted in natural conditions in the Rhodopes Mountains experimental field where the survival rate was 68%, after winter has passed. In April, vigorous vegetation was observed with formation of sprouts, floweres, seeds and rhizomes like plants in their natural environment.

Genetic stability of *in vitro* regenerated plants is very important for micropropagation aiming production of elite plant material or conservation of the species. Chromosome number in the root tip cells of *in vitro* regenerants of *Rhodiola rosea* was examined. All the plantlets though obtained on different media had 22 chromosomes which number was identical with the diploid chromosome number of 2n = 22 of the wild plant. These results indicate that the regeneration schemes developed by authors [197] favor stability of the initial karyotype. This fact is very important for the purposes of restoration of the species and for creating nurseries and fields of Golden root serving the pharmacological needs.

Another very important fact is the ability of *in vitro* obtained plants to synthesize salidroside what was confirmed by the analysis of one and two years old regenerants. Salidroside content

in all the samples taken from the roots of regenerants reintroduced in nature was higher than those in plants, which developed from seeds in the mountains [198].

Roots and rhizome from one year old plant regenerants growing in the green house have lower salidroside content compared to the plants growing in the experimental field in the mountains at an altitude of 560 m. However, at the same conditions high levels of rosavin 3.2 % and 3.3 % were detected in green house plants and in mountain plants, respectively (unpublished data). Golden root extracts used in major part of the clinical research are standardized to 3.0 % of rosavins and 0.8% salidroside, which is a ratio of 3:1. This ratio was 10.75:1 in the experiments of the authors (unpublished data) for green house one year old regenerants. Similarly one and three years old regenerants growing in the mountains had higher portion of rosavins compared to salidroside (1 : 8.6 and 1 : 3.75, respectively) which was very positive fact (unpublished data)

Recently replanting of *Rhodiola rosea* regenerants in natural conditions was reported from other authors, too [199] but unlike the previous report [43] reintroduced regenerants differ morphologically. Several types of explants and nutrient media were used to reveal the morphogenic potential suitable for elaborating schemes for micropropagation [199]. The most efficient combinations were when explants from shoot nodes and apices were cultured on MS medium containing 2.0 mg/l NAA, followed by hormone free MS, then KN (1 mg/l kinetin and 0.5 mg/l NAA), and AZ (0.2 mg/l IAA and 2 mg/l zeatin). The *in vitro* generated neo plantlets reached survival rate over 90% after transfer to septic environment in a hydroponic system for 5-7 days. After acclimatization, the regenerants were potted into soil until the first summer when they were transferred to their native habitat (at 1750 m altitude in Ceahlău Mountains, Romania). During the next summer about 73.5 % of the few dozens of reintroduced regenerants survived. This percentage dropped at 57 % during the third year. It was observed that the *in vitro* regenerants of *Rh. rosea* developing in their natural habitat differed in leaf color (light green), compared to the native individuals of this region (green- grey).

For the first time an original protocol for *in vitro* micropropagation of *Rhodiola rosea* in a RITA bioreactor system was reported [200]. Three clones were obtained from *in vitro* germinated seedlings of wild Finland golden root. Stimulation of organogenesis was studied using thidiazuron and zeatin. Two to four μM thidiazuron stimulated shoot induction but inhibited shoot growth while 1-2 μM zeatin favored shoot growth and leaf number per shoot. Multiplication rate of the clones differed significantly but the most efficient was obtained on solidified medium enriched with 2 μM zeatin. In the bioreactor 0.5 μM thidiazuron maintained rapid shoot proliferation but induced hyperhydricity at higher concentrations. However, hyperhydricity was abolished when shoots were transferred for 4 weeks on gelled medium enriched with 1-2 μM zeatin. Shoots formed roots for 5-6 weeks on medium without phytohormones. Regenerants transferred to soil in the green house survived at high rate (85–90%) and after acclimatization had normal shoot and leaf morphology.

After establishment of reliable *Rhodiola* *in vitro* cultures, research has continued for their implementation for practical use like production of valuable secondary metabolites in bioreactors, for biotransformation, for manipulation of the metabolic pathways and metabolic engineering. Biotransformation is a key mechanism to increase production of the biologically

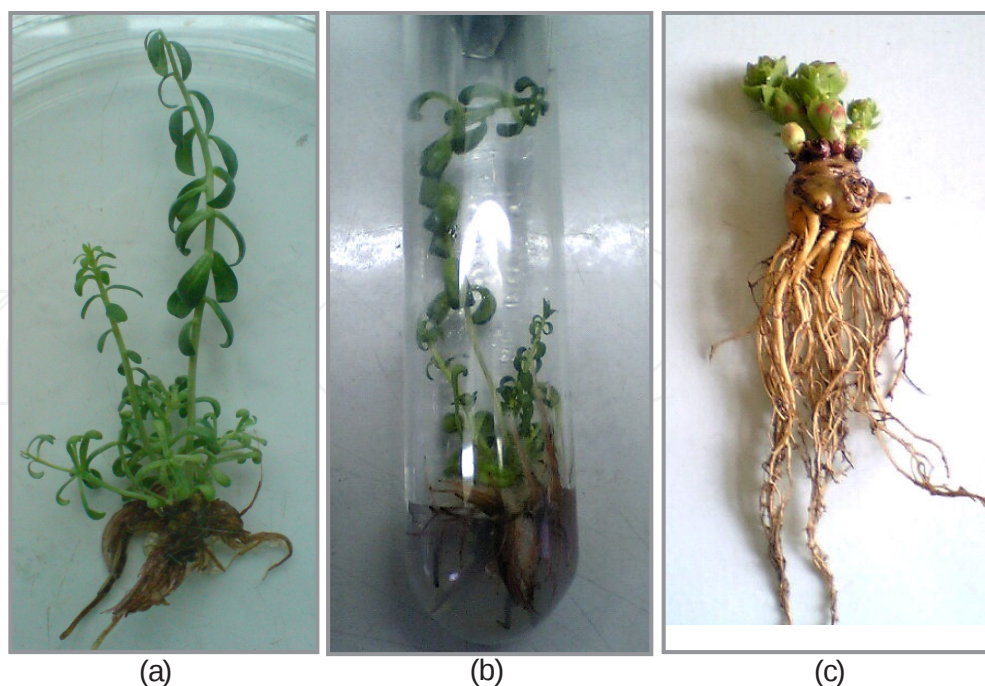


Figure 3. *In vitro* regenerants of *Rhodiola rosea* Bulgarian ecotype: (a) and (b) – propagated plants on MS medium enriched zeatin; (c) – two years old regenerants growing in green house.

active compounds in callus cultures. There are few reports on golden root callus cultures with accompanying analysis of their biologically active metabolites and description of the parameters for their efficient synthesis *in vitro*. The first attempts dated a decade ago [201]. Callus was induced on leaf explants of *Rhodiola rosea* and transferred into MS liquid medium. Thus obtained suspension culture was used to study the possibility to increase synthesis of rosavin and other cinnamyl glycosides. In the cells for about 3 days, more than 90% of the added transcinnamyl alcohol (optimal concentration of 2.5 mM) was transformed into various unidentified products. However, one of them, 3-phenyl-2-propenyl-O-(6'-O- α '-L-arabinoryranosyl)- β -D-glucopyranoside, found in the intracellular spaces, both of green and yellow strains of cell cultures, was defined as potential rozavin by very precise methods.

Biotransformation was used for increasing of biologically active substances production in callus culture in *Rhodiola rosea*. The effect of different precursors of biologically active substances on the biomass and the metabolite production was studied in *Rhodiola rosea* compact callus aggregates in liquid medium [202, 203]. Cinnamyl alcohol concentrations up to 0.1 mM in media did not bring to a significant deviation from the control; 2 to 5 mM changed slightly callus color from dark to light green. In these cultures rosin content was elevated to 1.25 % dry weight while rosavin was 0.083% dry weight. Cinnamyl alcohol induced synthesis of four new products, too. Tyrosol from 0.05 mM and 2 mM did not influence callus growth while concentrations of 3 mM up to 9 mM caused decrease in biomass production. Two mM of tyrosol were the optimal levels for salidroside production reaching 2.72 % dry weight. Addition of glucose had no positive effect on salidroside accumulation but doubled the rosin production.

Callus tissues cultivated on solid media could produce active substances characteristic for the species [204] *Rh. rosea*. Addition of yeast extract in the media doubled salidroside content (from 0.8 % to 1.4) and was twice as high as in five-year-old roots of the intact plants. In the later experiments [205] *Rh. rosea* callus induced from axillary buds or from seedling hypocotyls transformed exogenous cinnamyl alcohol into rosin. However, the biotransformation process was more efficient in the hypocotyl callus where the application of 2.5 mM cinnamyl alcohol resulted in the increase of rosin content up to 1056.183 mg/100 g on solid medium and 776.330 mg/100 g in liquid medium. Callus tissue obtained from axillary buds and treated in the same way produced rosavin in a higher concentration of 92.801 mg/100 g and reached 20% of the amount produced by roots [206].

Krajewska-Patan et al. and György et al. [205, 202, 203] obtained and maintained callus from *Rh. rosea* in liquid medium adding different precursors of the biologically active substances to increase the synthesis of the substances from the main biologically active complex.

The same Bulgarian group successfully established callus cultures, too [207]. Induction of callogenesis was achieved from leaf explants, isolated from *in vitro* propagated plants, on MS media enriched with BAP in concentration from 0.5 mg/l to 2.0 mg/l; 2-iP—0.3 and 3.0 mg/l; 2,4-D—from 0.1 to 2.0 mg/l; IAA—0.2, 0.3 and 1.0 mg/l; NAA—0.5, 1.0, 1.5 mg/l; glutamine—150 mg/l and casein hydrolysate 1000 mg/l. The highest response of 62.85 % and 73.17 % formation of callus was observed on two media, both containing 1 mg/l BAP and either 1 mg/l or 0.5 mg/l 2,4-D (Figure 4 a, b, c, d, e, f, g, h). The authors observed (unpublished data) that when calli were cultured on media with the same phytohormones as mentioned above but with higher content of sucrose (3 % instead of 2 %) the induction of callogenesis was several folds lower and variations in callus structure and color were noted. Sucrose concentration influenced synthesis of biologically active substances. Phytochemical analysis revealed that at 2 % sucrose in the medium salidroside and rosavins were not detected in the calli (unpublished data)

Similar investigations were performed with other *Rhodiola* species. *Rh. sachalinensis* calli cultured with 5% sucrose produced high salidroside content (0.41 % on the basis of dry wt) than normal root (0.17 %) [208]. A compact callus aggregate strain and culturing system for high yield salidroside production was established in *Rhodiola sachalinensis* [209].

Organogenic callus was obtained from leaves with efficiency of 88.33 % [210]. Among the yellow, green, and red colored calli, only green callus formed buds though with poor efficiency. Despite this, regenerated plantlets were rooted on half strength MS medium. Experiments with *Rhodiola sachalinensis* proved that cryopreservation of calli is possible followed by successful recovery of fresh and green tissues for 6 weeks. Isolation of protoplasts was also reported for this species [211].

in vitro cultures were obtained from *Rh. crenulata*, *Rh. yunnanensis*, *Rh. fastigata* [212, 213] and *Rh. quadrifida* [214] proving the role and interactions of the explant type, genotype and phytohormones for the efficiency of *in vitro* response and regeneration was also function of the genotype and the phytohormones. The authors underlined the role of 2,4-D and BA for production of biologically active substances. Similar observations about the role of the explant,

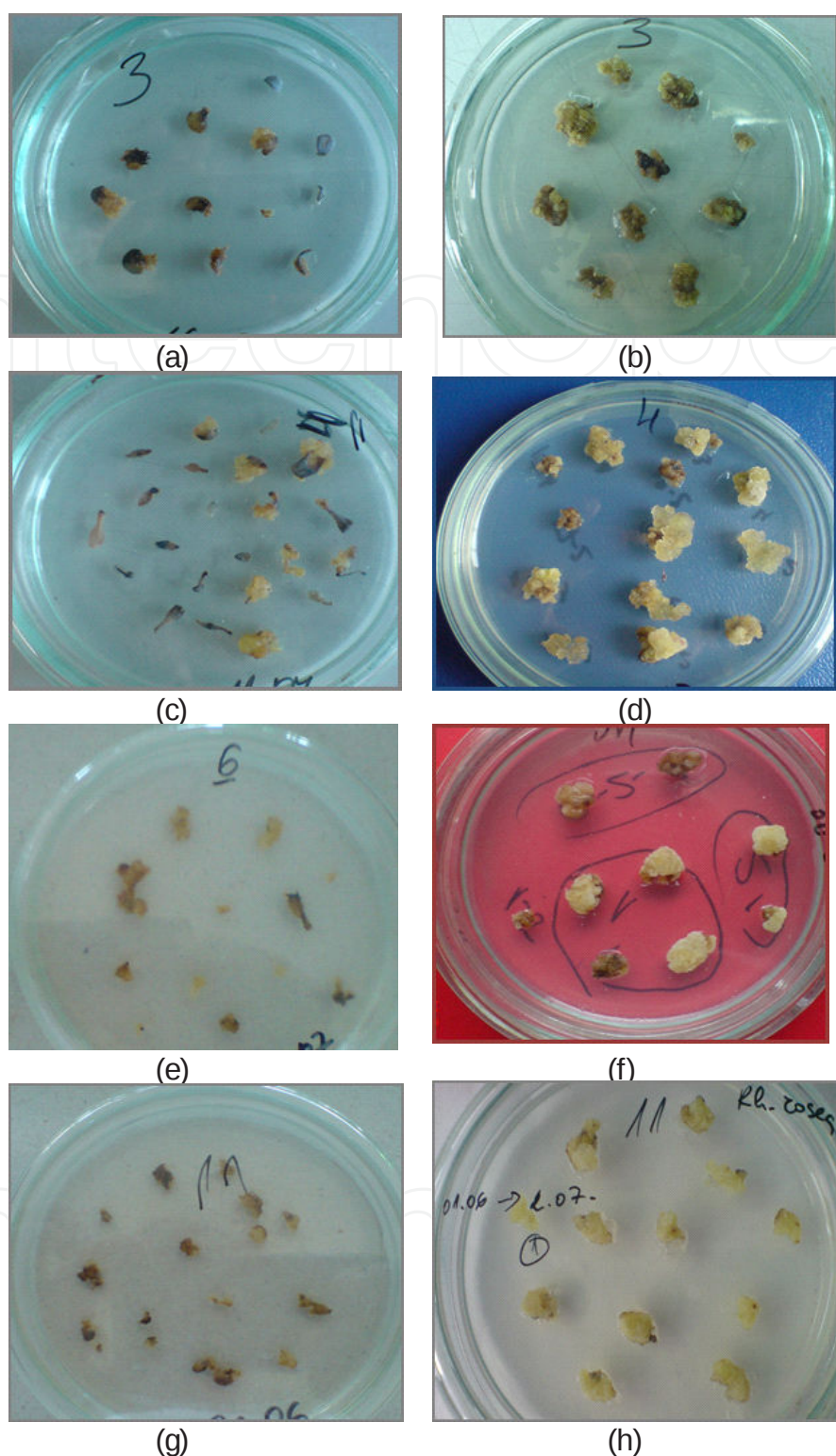


Figure 4. Various callus cultures induced on MS basal medium enriched with: (a) – BAP (1 mg/l), 2,4-D (1 mg/l) and 3% sucrose; (b) – BAP (1 mg/l), 2,4-D (1 mg/l) and 2% sucrose; (c) – BAP (1 mg/l), 2,4-D (0.5 mg/l) and 3% sucrose; (d) – BAP (1 mg/l), 2,4-D (0.5 mg/l) and 2% sucrose; (e) – BAP (1 mg/l), 2,4-D – 1 mg/l, Casein hydrolysate 1000 mg/l and 3 % sucrose; (f) – BAP (1 mg/l), 2,4-D – 1 mg/l, Casein hydrolysate 1000 mg/l and 2 % sucrose (g) – BAP (1 mg/l), NAA (0.5 mg/l), Casein hydrolysate 1000 mg/l and 3% sucrose; (h) BAP (1 mg/l), NAA (0.5 mg/l), Casein hydrolysate 1000 mg/l and 3% sucrose;

the temperature of cultivation and the pretreatment duration on salidroside synthesis in *Rhodiola kirilowii* callus were made by others [215].

Genetic transformation opens new perspectives for production of biologically active compounds. Hairy roots induced by *Agrobacterium rhizogenes* grow faster accumulating greater biological material. Genetic transformation of *Rhodiola sachalinensis* was performed with *Agrobacterium rhizogenes* [216, 217]. The authors studied conditions for high salidroside production (the major compounds from the roots of *Rhodiola sachalinensis*) when precursors (tyrosol, tyrosine, and phenylalanine) and elicitors (*Aspergillus niger*, *Coriolus versicolor*, and *Ganoderma lucidum*) were added into the medium. For high salidroside production, the optimal light intensity, pH value and nitrogen levels were determined, too. The optimal concentration for the elicitor was 0.05 mg/l while the optimal concentration of the precursor was 1 mmol/l. The 1000 lx scatter light, pH 4.5 - 4.8, and nitrogen ($\text{NH}_4^+:\text{NO}_3^- = 1:1$) concentration of 80 mmol/l were the optimal condition for salidroside production. Authors conclude that hairy roots can be used as alternative material for the production of secondary metabolites of pharmaceutical value in *Rhodiola*.

Examples, given here, though covering a small part of the enormous and tedious work on medicinal plants, and more particularly on representatives of the genera of *Gentiana*, *Leucojum* and *Rhodiola*, which are protected in Bulgaria, could give impression on the potential of different spheres of plant biotechnology (Table 1). The most promising ones being *in vitro* clonal propagation of endangered species to create *in vitro* and *ex situ* collections, and for obtaining of raw material and valuable compounds (Figure 5).

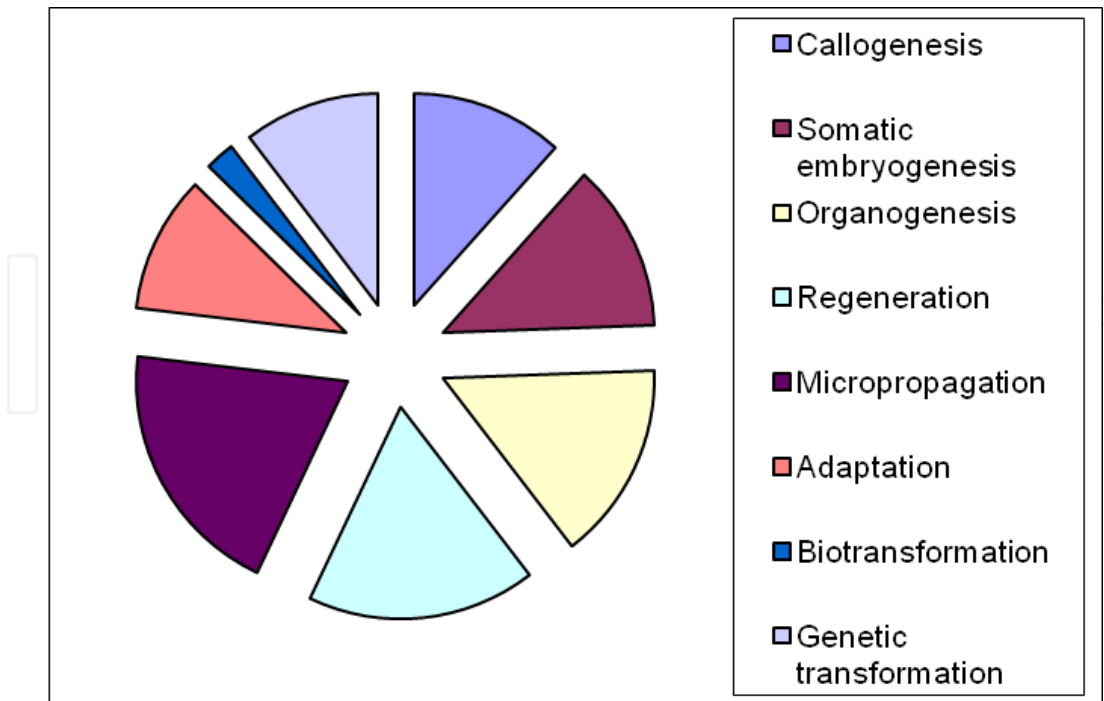


Figure 5. Relative share of the achievements in different spheres of biotechnology in the three genera: *Gentiana*, *Rhodiola*, *Leucojum*.

Plant Species	Callusogenesis	Somatic embryogenesis	Organogenesis	Regeneration	Micropropagation	Adaptation	Biotransformation	Genetic transformation
<i>G. lutea</i>	yes	yes	Yes	yes	yes	yes		yes
<i>G. kurroo</i>	yes	yes	Yes	yes	yes	yes		yes
<i>G. cruciata</i>		yes	Yes	yes	yes			yes
<i>G. pannonica</i>		yes						
<i>G. punctata</i>			Yes	yes	yes	yes		yes
<i>G. straminea</i> Maxim.		yes						
<i>G. crassicaulis</i> ,		yes						
<i>G. dinarica</i> Beck,			Yes	yes	yes	yes		
<i>G. corymbifera</i> ,			Yes	yes	yes			
<i>G. pneumonanthe</i>		yes			yes	yes		
<i>G. purpurea</i> ,		yes	Yes	yes				yes
<i>G. davidii</i> var. <i>Formosana</i>		yes						
<i>G. scabra</i>	yes			yes				
<i>G. acaulis</i>			Yes	yes				yes
<i>G. tibetica</i>		yes						
<i>G. dahuria</i>			Yes	yes	yes	yes		yes
<i>G. triflora</i>					yes			
<i>G. ligularia</i>					yes			
<i>G. cerina</i>					yes	yes		
<i>G. asclepiadea</i>			Yes	yes				
<i>Rh. rosea</i>	yes		Yes	yes	yes	yes	yes	
<i>Rh. crenulata</i>					yes			
<i>Rh. kirilowii</i>	yes							
<i>Rh. quadrifida</i>	yes							
<i>Rh. sachalinensis</i>	yes				yes			yes
<i>Rh. yunnanensis</i>					yes			
<i>Rh. iremelica</i>	yes		Yes	yes				
<i>Rh. fastigata</i>					yes			
<i>Rhodiola coccinea</i>	yes			yes				
<i>Leucojum aestivum</i>	yes	yes	Yes	yes	yes	yes	yes	yes

Table 1. Examples of biotechnological achievements in *Gentiana*, *Rhodiola* and *Leucojum* species.

9. Conclusions

Presented data and results in this chapter aimed at enlightening the potential of plant biotechnologies in protection of valuable plant species, including the medicinal ones, which have

become rare or are close to extinction as a result of the intensive industrialization, urban economy and climatic changes. One of the measures for overcoming this global problem could be the cultivation of valuable medicinal plants in experimental conditions. For this purpose along with the traditional methods for cultivation fields and nurseries, “green” biotechnologies can be used. Many scientists have realized that plant biotechnology is an important tool for multiplication and conservation of the endangered and rare populations of medicinal plants. Using environmental friendly *in vitro* technologies a great number of identical plants, can be propagated, regenerated and transferred back in nature thus restoring and expanding wild habitats. From another hand, the areas of the medicinal plants will be less subjected to vulnerable exploitation if the valuable raw material could be obtained by alternative means. In this sense by micropropagation of plants, enormous amounts of biomass can be produced continuously and/or for short period of time. In addition, production of biologically active substances in laboratory conditions contributes to less utilization of the natural resources and thus protecting the species. The fact that *in vitro* cultures, cells, tissues, organs and plantlets can produce metabolites, specific for the intact donor plant, is of tremendous importance for production of desired compounds. Development of more sophisticated instrumentation and original approaches allowing biotransformation and metabolic engineering is a revolutionary step for high technological production of valuable substances and biologically active compounds demanded from the food, nutraceutical, pharmaceutical and cosmetic industries.

Nomenclature

MS – Murashige and Skoog medium, 1962; BAP – N⁶-benzylaminopurine; IAA – Indolyl-3-acetic acid; 2-iP – 6-(y,y-dimethylallyl amino) purine; 2,4-D – 2,4- dichlorophenoxyacetic acid; NAA - α - naphthyl acetic acid; TDZ – Thidiazuron; Kin – Kinetin; GA₃ – Gibberellic acid; IBA – Indole 3-butyric acid

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