

Institute of Organic Chemistry with Center of Phytochemistry-
Bulgarian Academy of Sciences

Habilitation contributions

**of Kalina Danova, Senior Assistant professor, Laboratory Chemistry of Natural Compounds,
Institute of Organic Chemistry with Centre of Phytochemistry**

Sofia

2019-07-31

For application in the procedure for Associate professor in professional field 4.2. Chemical sciences, scientific discipline "Bioorganic chemistry, chemistry of natural and physiologically active compounds" for the needs of Laboratory "Chemistry of Natural Compounds", announced in the Bulgarian State Official Journal, 43/31.05.2019

Contents

1. Introduction.....	2
1.1. Necessity of conservation of biodiversity of medicinal and aromatic plants in Bulgaria	2
1.2. Necessity of investigation of the biosynthetic capacity medicinal and aromatic plants in biotechnological conditions.	3
1.3. Ecological role and medicinal value of <i>Hippophae rhamnoides</i> L.	4
1.4. Medicinal properties of <i>Clinopodium vulgare</i>	5
1.5. Medicinal properties and tissue culture development of <i>Hypericum</i> species	6
1.6. Medicinal properties, essential oil variability and tissue culture development of <i>Artemisia alba</i> Turra.	7
2. Main scientific contributions of the candidate	7
2.1. Results of the work on <i>Hippophae rhamnoides</i> – (B-Q2-1).....	8
2.1.1. Comparison of flavonoid content of <i>H. rhamnoides</i> collected from different natural conditions.	8
2.1.2. <i>In vitro</i> culture development.....	9
2.1.3. Revision of the natural populations and the conservation status of the species in Bulgaria.	9
2.2. Results of the work on <i>Clinopodium vulgare</i> – (B-Q3-3)	10
2.2.1. Establishment of shoot cultures.....	10
2.2.2. Extraction and analysis of nitrogen oxide radical scavenging activity.	10
2.3. Results of the work on <i>Hypericum</i> species.....	10
2.3.1. Establishment of shoot cultures of <i>Hypericum</i> species.....	10
2.3.2. Assessment of radical scavenging activity of hypericin producing <i>Hypericum</i> species (B-Q3-3).....	10
2.3.3. Assesment of the relations between enzymatic activity and polyphenolics productivity of hypericin non-producing <i>H. calycinum</i> (B-Q3-4).....	11
2.4. Results of the work on <i>Artemisia alba</i> Turra.....	11
2.4.1. Tissue culture development	11
2.4.2. Characterization of the obtained plant material.....	12
2.4.3. Characterization of secondary metabolite content of the <i>in vitro</i> cultured plant.....	12
2.4.4. Characterization of endogenous cytokinin production and chloroplast architecture in the two <i>Artemisia alba</i> morphotypes <i>in vitro</i>	12
3. Outline of future perspectives of the scientific topic within the next three years	13
3.1. Work related to the differentiated lines of <i>in vitro</i> cultivated plants	13
3.2. Work on genetically non-transfomed root cultures of the cultivated plants	14
3.3. Work on the non-differentiated lines of plants grown in liquid media	15

4. Bibliography.....	16
4.1. Bibliography of literature sources of other authors used in the Introduction.....	16
4.2. Bibliography of “B” publications of the candidate included in the habilitation contributions..	17
4.3. Bibliography of “Г” publications of the candidate in addition to the habilitation contributions	18
4.4. Other publications of the candidate	19

1. Introduction

1.1. Necessity of conservation of biodiversity of medicinal and aromatic plants in Bulgaria

Bulgaria is located in the Southeast of Europe, in the central part of the Balkan Peninsula. The country comprises parts of 3 bio-geographic regions: alp, Black sea and continental. The various relief, geology and specific microclimatic conditions determine the rich diversity of species, populations and natural habitats, many with conservation significance in Bulgaria. Considering biodiversity richness, the country ranks among the first places in Europe (CBD, 2005). Bulgarian vascular flora comprises of about 3900 species (Petrova and Vladimirov, 2007).

According to the latest performed botanical survey, the Red List of the Bulgarian vascular plants (threat categories) comprises 801 species (20.5 % of the total flora), of which one Extinct (EX), 12 Regionally Extinct (RE), 208 Critically Endangered (CR), 297 Endangered (EN), 204 Vulnerable (VU), and 79 Near Threatened (NT). The list of other evaluated species comprises 96 taxa, of which, 53 Data Deficient (DD) and 43 Least Concern (LC) (Petrova and Vladimirov, 2009).

Area protection of habitat types and the habitats of species with national and European significance are realized by a National Ecological Network. Protected areas are included therein, declared in compliance with the Bulgarian specialized legislation as well as protected sites, part of the European Ecological Network NATURA 2000, helping to preserve ecosystems and biodiversity. Still biodiversity is not sufficiently valued as a decisive factor for sustainable development and main criteria for wealth and development of the society, as a result of which in some regions of the country significant damages have been caused and special efforts will be necessary for recuperation. In scientific aspect, a certain potential is supposed to be redirected toward newer and more actual trends of the policy on the

biodiversity domain such as: evaluation of the ecosystem services, economical aspects of the biodiversity and adaptations to climatic changes (CBD, 2005).

There already exist living collections and seed banks of endangered plant species in the specialized scientific departments and botanical gardens. However, *ex situ* conservation facilities are still necessary to bolster and complement *in situ* conservation programs (IPCC, 2007).

1.2. Necessity of investigation of the biosynthetic capacity medicinal and aromatic plants in biotechnological conditions.

Although plant metabolic profile is genetically pre-determined, its qualitative and quantitative characteristics are quite dynamic and can vary strikingly depending on the complex interrelations of the plant organism with its surrounding environment. In addition, plant secondary metabolites production, accumulation and translocation are dependent on the presence of highly specialized anatomical structures within the plant organism. Therefore secondary metabolites content is strongly affected by the developmental patterns and morphogenesis of the plant individual. Therefore, the controlled modification of the parameters of the plant's environment, as it is in the *in vitro* system of tissue culture conditions, makes it possible to target the production of plant biomass with desired properties (**I-Book Ch-1, 3**).

The commercial yield of plant secondary metabolites by plant cell tissue and organ cultures is still limited as compared to the wide market application of plant biotechnology in agriculture and ornamental plants breeding. Nevertheless, numerous successful examples show the prospective for the development of this field (**I-Book Ch-3**).

First attempts for the production of secondary metabolites by *in vitro* cultivated plant tissues were performed by the Charles Pfizer Co Company in 1950 (Lombardino 2000; Dias et al. 2016). Production, experimented on *in vitro* cultured water-melon tissue turned unsuccessful. Further on, the first patent obtained in this area was by Routian and Nickell as assignors to Chas. Pizer & Co., Inc., New York, N. Y., a corporation of Delaware (Routian and Nickell 1956). The invention consisted of “a method for cultivating plant tissue under submerged, aerated conditions in liquid culture” and was “also concerned with the production of useful materials by this method”. Tissues of several plant species were included, designated as “tissues of those plants classified above the Thallophytes in systematic botany” (including *Rumex acetosa* L., Sweet clover, *Agave toumeyana*, Sunflower stem and petiole, Periwinkle,

Tobacco, *Opuntia* cactus, *Datura* and avocado). The delivery of “vitamins, steroids, alkaloids of various types, antimicrobial agents, sugars, enzymes, organic acids, aromatic materials, and so forth” was defined.

Further development after 1978 in the field led to feasible biotechnological production of secondary metabolites in Germany and Japan (Loyola-Vargas and Vázquez-Flota 2006; Dias et al. 2016). Thus, now-a-days the number of patents, involving plant cell cultures has risen to the impressive number of 28 000, with companies, applying plant cell cultures or hairy roots for the production of ingredient for cosmetics, food and pharmaceutical industry has been intensively expanding (Ochoa-Villarreal et al. 2016).

The comprehension of the role of developmental and morphogenetic features for the biogenesis of plant secondary metabolites is essential for optimization of secondary metabolite productivity *in vitro*. In addition, complex and interdisciplinary research is needed in order to identify key factors, responsible for the production of certain secondary metabolite classes. Obtaining the knowledge and understanding these factors would allow for the biotechnological production of desired secondary metabolites to be realized by scientifically based approach utilizing the indigenous biosynthetic capacity of plant species without performing genetic manipulations.

1.3. Ecological role and medicinal value of *Hippophae rhamnoides* L.

Hippophae rhamnoides L., a member of Eleagnaceae, is thorny nitrogen fixing deciduous shrub or small tree. The plant occurs naturally throughout a wide area in Europe and Asia (Roussi , 1971) mainly at sandy sea shores and valleys of mountainous rivers at altitudes from the sea level to 2000m and higher (mainly in Northern Caucasus and Pamir). The Southern boundary of its distribution range passes through Bulgaria where the species is known since the beginning of the 20th century, being distributed in the surroundings of Varna (B-Q2-1).

The plant has been known for its valuable medicinal properties since ancient times. It is mentioned in the writings of ancient Greek scholars such as Dioskorid and Theophrastus. Since the ancient Tibetans started using Sea buckthorn more than one thousand years ago, hundreds of Asian traditional recipes have been developed and carried on through generations. Sea buckthorn is a phytoprotective agent to human health. All parts of the plant are considered to be a good source of a large number of bioactive substances. It has antioxidant, anti-ulcerogenic and hepato-protective actions, and its berry oil is reported to

suppress platelet aggregation (Li and Wang, 1998; Gao et al. 2000; Suleyman et al. 2001). In a radioprotection study, a dose of 30 mg/kg body weight of herbal preparation of the whole berries of the plant was reported to render 82% survival as compared to no survival in irradiated control, as this effect was attributed to the free radical scavenging, acceleration of stem cell proliferation and immune-stimulation of the extract. Further investigation of this action revealed, that the berries preparation at a concentration of 120µg/ml or more induced a strong compaction of chromatin, which made the nuclei resistant even to a radiation dose of 1000Gy (Kumar et al. 2002).

The species has an important ecological role as well, as it is effective for soil erosion control and reclamation of marginal land. The plant has an extensive root system and can grow effectively in marginal soils since nitrogen-fixing actinomycetes can form a symbiotic relationship with the roots (Akkermans et al. 1983). Typical contributions of fixed nitrogen by Hippophae associations are 27 to 179 kg of N ha⁻¹ year⁻¹ (Baker and Mullin 1992).

The valuable medicinal and ecological properties of the species, as well as the fact that it is native to the Bulgarian flora impose the necessity of its popularization and practical utilization. The Endangered status of the plant species demands serious conservation measures for its preservation.

1.4. Medicinal properties of *Clinopodium vulgare*

Clinopodium vulgare L. is commonly used in Bulgarian folk medicine for the treatment of irritated skin, mastitis- and prostatitis-related swelling, as well as for some disorders accompanied by significant degree of inflammation (e.g. gastric ulcers, diabetes, and cancer). In addition, anti-inflammatory, strong free radical scavenging and antitumor activities of the aqueous extract of this plant have also been reported (Dzhambazov et al., 2002; Burk et al., 2009). The species falls under the scope of the Bulgarian Law for Medicinal Plants (2017). There are no reports in available literature on tissue culture development of this species.

1.5. Medicinal properties and tissue culture development of *Hypericum* species

The *Hypericum* genus comprises over 484 species grouped in 36 sections. Species are either naturally occurring or introduced to every continent on the world excluding Antarctica, their habitus varying from trees to herbaceous individuals. Based on the rich ethnobotanical data of the traditional utilization of its representatives, the *Hypericum* genus is probably one of the most widely studied medicinal plants taxon regarding its secondary metabolite content and pharmacological properties (Γ-Book_Ch-1 and 2 and references cited within).

Hypericum perforatum L. is the most widely utilized representative of the genus. It has been used since the 1st century A.D. It is native to Europe, North Africa and Asia, but also naturalized to North America. The broad spectrum of activities of *H. perforatum* (St John's wort) is determined by the specific complexity of the *Herba Hyperici* extract and the potential additive effect, synergism or even possible antagonism between its different components. Research has led to elucidation of the most important biologically active substances in the plant – polyphenolic compounds, flavonoids, naphthodianthrones and phloroglucinols, terpenes. These constituents were shown to possess antidepressive, antitumor, antiviral and antibiotic activity. Research interest towards the plant has been incessant throughout the years (Γ-Book_Ch-1 and references cited within).

Regarding the biosynthetic potential of the different representatives of the genus, in spite of certain similarity of its main phytochemical constituents, remarkable intra- and interspecific differences of the quantitative and qualitative characteristics have been recorded, between populations of the same species from different locations and within the individual developmental phases of the given individual. An interesting feature, regarding the biosynthetic potential of the different representatives of the genus is that the evolutionary old sections are characterized by a lack of hypericins production and higher phloroglucinol levels, as compared with the evolutionary more developed sections. In addition, literature surveys have shown that the evolutionary more developed *Hypericum* species possess an increased capacity of hypericins production as compared to the “lower” sections of the genus (Γ-Book_Ch-2 and references cited therein).

The phytochemical studies of *Hypericum* species indigenous to the Balkan region have provided evidence for the phytochemical potential of these accessions as novel sources of phytopharmaceuticals characteristic for the genus (Γ-Book_Ch-2 and references cited

therein). Bulgarian flora provides the richness of wild accessions of 22 *Hypericum* species, of them 5 Balkan and one Bulgarian endemic species (the latter, however, extinct). Sixteen of these species (including the extinct one) belong to sections which are evolutionary more developed than Section *Hypericum* (to which the most widely studied *H. perforatum* belongs).

Throughout the years the genus has been an incessant object also of scientific interest in terms of secondary metabolites production in plant cell tissue and organ culture (Γ-Book_Ch-2 and references cited within). Interestingly, with only few exceptions, plant cell tissue and organ culture development have been focused on *Hypericum perforatum*, as well as other species of the *Hypericum* Section of the genus.

1.6. Medicinal properties, essential oil variability and tissue culture development of *Artemisia alba* Turra.

Artemisia alba Turra is a fragrant shrub distributed in Southern Europe. Although not as prominent as *A. annua* for producing the anti-malarian artemisinin, the species has been reported to exert spasmolytic, antimicrobial and anti-diabetes activity, especially regarding its essential oil properties (Ronse and De Pooter 1990; Stojanovic et al. 2000). However, surveys have revealed a great variability of its terpenoid profile, attributed by different authors to environmental conditions, geographic distribution and/or to genetic factors (Radulović and Blagojević 2010 and ref. cited within). There is scarce information on tissue culture development for this species. Available data concern comparison of the essential oils obtained from *ex vitro* and *in situ* samples of *A. alba* (Ronse and De Pooter 1990) and tissue culture initiation with conservational purposes (Holobiuc and Blindu 2006 - 2007).

2. Main scientific contributions of the candidate

The scientific contributions summarized below have been published in seven works included in the habilitation contributions (enlisted as “B” below); in addition the bibliography below contains eleven publications and three book chapters in addition to the habilitation contributions (enlisted as “T”), as well as 25 publications, enlisted as “other publications” of the candidate.

The scientific contributions enlisted below encompass three main aspects:

- ❖ Conservational aspect.

The experimental work, described in the habilitation contributions below, has led to the setting and elaboration of facilities for plant cell tissue and organ culture development of medicinal and aromatic plants in the Institute of Organic Chemistry with Centre of Phytochemistry-BAS. This makes it possible to maintain a collection of slow-growth cultures of medicinal and aromatic plants, collected from Bulgaria and also from other locations worldwide.

❖ Fundamental aspect.

The maintenance of a rich collection of medicinal and aromatic plants at the facilities of IOCCP-BAS, allows for implementation of rapid, effective and feed-back based research from the cultivation to characterization of growth, development and physiological status to secondary metabolite characterization.

The conducted experiments and obtained results allow for the clarification of key aspects for the better understanding factors affecting the different classes of secondary metabolites under investigation. This determines the flexibility of the approach to optimize biotechnological tools for targeting secondary metabolite production by utilization of the indigenous biosynthetic capacity of the species without performing genetic manipulations.

❖ Applied aspect

The obtained concrete results have led to obtaining of concrete biological *in vitro* systems of the studied species, producing plant material with defined properties. The secondary metabolites obtained are with known phytoterapeutical properties; however the *in vitro* sources for their extraction are new and are present at the facilities of IOCCP-BAS. This allows for the potential of for the practical application of the obtained processes for biotechnological of up-scale of the production of desired secondary metabolites without performing genetic manipulations which is a necessary prerequisite when practical application is sought in the field of cosmetics, food industry and pharmacy.

2.1. Results of the work on *Hippophae rhamnoides* – (B-Q2-1)

2.1.1. Comparison of flavonoid content of *H. rhamnoides* collected from different natural conditions.

The analysis was performed by means of colorimetric assay of the content of total flavonoid compounds, as described in (B-Q2-1). Plant samples from material growing in natural and introduced populations were compared. Samples, collected at the site of its

natural habitat at Pasha Dere near Varna contained 12.75 mg/g DW (1.28%), and samples of the *in situ* collection of the Botanical Garden-BAS, Sofia – 7.48 mg/g DW (0.75%). For comparison other authors report quantities of total flavonoids 0.83 – 2.0%, the highest value being at elite selections; and others - 0.87mg/g DW – 38.88 mg/g DW (references cited in B-Q2-1).

2.1.2. *In vitro* culture development.

Shoot cultures of the species were established from surface sterilized stem explants, collected from the Botanical Garden of the Bulgarian Academy of Sciences, Sofia. The top most parts of young offsets of the freshly collected branches were cut into segments approximately 1.5 cm in length, containing one to four nodules; the adjoining petioles and laminae basis were kept intact as to preserve the axillary buds. Dormancy of axillary buds was broken by means of the addition of different concentrations of benzyl adenine (BA) to the medium and osmotic pressure was decreased by means of reducing the content of macro-salts of the Murashige and Skoog medium formula.

2.1.3. Revision of the natural populations and the conservation status of the species in Bulgaria.

The investigation was done with the kind cooperation of Assoc. Prof. Antoaneta Petrova, Director of the Botanical garden of BAS. Visual observation of photographic material as well as on-site inspection on the changes of the population were done, which is the only practical possibility, because of the prevailing type of the habitat of the species in Bulgaria (steep, almost vertically inclining friable sea banks). The conservational evaluation was made according to the methodology of IUCN (2001), applied on a regional level as described in (B-Q2-1). Based on the evaluation of the status of the only remaining population of the species at Pasha Dere and the recommendations on its conservation made, the endangered status of the species was later changed from “Endangered” to “Critically endangered” (Petrova and Vladimirov, 2009).

2.2. Results of the work on *Clinopodium vulgare* – (B-Q3-3)

2.2.1. Establishment of shoot cultures.

Plant material was collected from its wild habitat in the Rila Mountain, Bulgaria. Shoot cultures were initiated by surface sterilized explants of the aerials of the plant. After induction of auxillary shoots in BA supplemented media, stock shoots were further maintained in plant growth regulators-free Murashige and Skoog medium.

2.2.2. Extraction and analysis of nitrogen oxide radical scavenging activity.

Methanolic extracts from the aerial parts of the plant were prepared and their NO-radical scavenging activity assessed in comparison with other *in vitro* cultivated plant material and vitamin C as control. The plant showed low activity of $SC_{50} = 3.45$, as compared with the referent compound – vitamin C with $SC_{50} = 0.26$ mg/ml.

2.3. Results of the work on *Hypericum* species

2.3.1. Establishment of shoot cultures of *Hypericum* species.

Shoot cultures of *H. tetrapterum*, *H. rumeliacum*, *H. richeri* and *H. calycinum* were established from surface sterilized plant material collected from the wild habitats of the species (for *H. calycinum*, whose natural habitats are with endangered status and are found in the Strandja Mountain, tissue cultures were initiated from aerial parts of the plant in garden conditions).

2.3.2. Assessment of radical scavenging activity of hypericin producing *Hypericum* species (B-Q3-3)

Methanolic extracts from the aerial parts of *H. tetrapterum*, *H. rumeliacum* and *H. richeri* were subjected to analysis for their NO-radical scavenging capacity and compared with the one of vitamin C as referent standard ($SD_{50} = 0.26$ mg/ml). It was established that the activity for *H. tetrapterum* was lower than the referent compound with $SD_{50} = 0.97$ mg/ml, the two species, belonging to the evolutionary more developed Drosocarpium section expressed higher activity of $SD_{50} = 0.18$ and 0.17 mg/ml for *H. rumeliacum* and *H. richeri*, respectively.

2.3.3. Assessment of the relations between enzymatic activity and polyphenolics productivity of hypericin non-producing *H. calycinum* (B-Q3-4).

For the purpose of the experiment plant growth regulators-free (PGR-free) control plants were maintained in the basic Murashige and Skoog basic medium. For the study of PGR effects benzyl adenine and indole-3-butyric acid were used. The following enzymatic activities: Phenylalanine ammonia lyase (PAL EC 4.3.1.24), glutathione reductase (GR EC 1.8.1.7), ascorbate peroxidase (APX EC 1.11.1.11); catalase (CAT EC 1.11.1.6) and superoxide dismutase (SOD, EC), non-enzymatic antioxidants (Ascorbate and dehydroascorbate, oxidized and reduced glutathione, as well as phenolic and flavonoid levels were estimated spectrophotometrically. The number of axillary shoots, as well as the leaf couples per shoot formed was recorded. Index of compactness (IC) was calculated as the number of leaf couples per 1 cm of shoot length. The obtained data was processed to distinguish between shoots with length lower and higher than 1 cm. The frequency of occurrence of each of the two types of shoot lengths was separately presented. PGR generally increased axillary shoot formation, but reduced IC and shoot length.

It was established that supplementation of PGR led to an increase of PAL and SOD, but inhibited CAT, GR, APX activities, as well as levels of polyphenolics and ratios of reduced/oxidised glutathione and ascorbate/dehydroascorbate in comparison with plant growth regulators-free control. Further on, it was established that elevation of IBA concentration stimulated axillary shoot formation and shoot length, but inhibited polyphenolic levels *in vitro*. These results demonstrated that stimulation/inhibition of leaf tissue in the plant corresponds to production of non-enzymatic antioxidants and physiological functioning of the enzymes CAT, GR, APX.

2.4. Results of the work on *Artemisia alba* Turra

2.4.1. Tissue culture development

Shoot cultures of the plant were initiated from surface sterilized aerial parts cuttings. Plant material was presented by Assoc. Prof. Ljuba Evstatieva from IBER-BAS. For modification of *in vitro* developmental patterns the PGR indole-3-butyric (IBA) acid benzyl adenine (BA) were added to the media (B-Q1-1).

2.4.2. Characterization of the obtained plant material

Two main morphotypes were achieved as a result – the PGR-free control and IBA supplemented plants displayed shoot and root development, and the plants where BA and IBA were combined, displayed lack of root formation and callusogenesis at the explant base (B-Q1-1).

2.4.3. Characterization of secondary metabolite content of the *in vitro* cultured plant

Comparison of the essential oils of the aerial parts of the two morphotypes revealed the presence of two main terpenoid profiles – oils with higher monoterpenoid/sesquiterpenoid ratio (plants with root development, irrespectively of the PGR concentrations) and oils with sesquiterpenoid domination (the group with inhibited rooting) (B-Q1-1). It was also established that unlike the aerials, monoterpenoid/sesquiterpenoid ratio of the roots expressed the opposite characteristics with sesquiterpenoids being prevalent in the root tissue (B-Q3-2). However, the qualitative identification of the terpenoid components of the underground parts showed that they sesquiterpenoids in the underground parts differed from the ones in the aerials (B-Q1-2).

Analysis of total phenolic content showed that inhibition of rooting significantly stimulated polyphenolic production *in vitro* (B-Q3-1).

Thus, as a general observation, it was established that the root suppressed *in vitro* system was characterized with elevated sesquiterpenoids and polyphenolics in its aerial parts.

2.4.4. Characterization of endogenous cytokinin production and chloroplast architecture in the two *Artemisia alba* morphotypes *in vitro*

Given the decisive role of roots for cytokinin biogenesis in the plant organism, further research was completed in order to assess the levels of endogenous cytokinins, as well as the chloroplast architecture in the samples of the different treatments. Inhibited rooting also resulted in a significant drop of endogenous isoprenoid CK bioactive-free bases and ribosides as well as CK *N*-glycoconjugates and in decreased *trans*-zeatin (*transZ*):*cis*-zeatin (*cisZ*) ratio in the aerials. Marked impairment of the structural organization of the photosynthetic apparatus and chloroplast architecture were also observed in samples with suppressed rooting.

It is well known that in the plant cell monoterpenoid and *trans*Ztype CKs biogenesis are spatially bound to plastids, while sesquiterpenoid and *cis*Z production are compartmented in the cytosol. The observed dependencies suggest an interplay between the biosynthesis of terpenoids and CK bioactive free bases and ribosides in *A. alba in vitro* via possible moderation of chloroplast structure has been hypothesized (B-Q1-2).

As a general conclusion, the dependencies obtained by this experimental design provide clues of the possibilities to target terpenoid biogenesis by means of inducing morphological changes of the *in vitro* cultivated plants without performing genetic manipulations.

3. Outline of future perspectives of the scientific topic within the next three years

Future plans are devoted to the continuation of the investigations which have already been started, following the scientific topics and aspects described above.

The experimental work will be conducted within the running projects and cooperation with the colleagues of the Bulgarian Academy of Sciences, Sofia University, as well as international partners who are already involved in the common research.

Opportunities will be sought for grant application on National and International programs for financial support of scientific research.

3.1. Work related to the differentiated lines of *in vitro* cultivated plants

As far as the shoot cultures of *Artemisia alba Turra* are concerned, additional research on the characterization of the two morphotypes obtained is foreseen.

Experiments aiming at the in-depth elucidation of the interrelations between the identified individual phenolic and flavonoid compounds and the physiological adaptation and endogenous stress hormones production *in vitro* will be conducted.

More detailed investigations on the effectiveness of the *in vitro* photosynthesis in the two morphotypes is also foreseen.

It is expected that the summary of the current results and planned research to shed more light on the interrelations between biogenetic pathways of these classes of secondary

metabolites and show possible interplay with the endogenous hormonal regulation and processes related to primary metabolism (such as photosynthesis) in this model plant species.

As a part of the implementation of project DN-09/11 (financed by the Bulgarian Scientific Fund), *in vitro* cultures of the medicinal plant ***Inula britannica***, **Asteraceae** are being cultivated. Preliminary research has been conducted, on relating patterns of growth and development to the production of sesquiterpene lactones and phenolic acids in the plant. Currently a study is being conducted on physiological status and photosynthetic pigments *in vitro*. Implementing the goals of the experiment is foreseen to allow for the optimization of *in vitro* system for the production of sesquiterpene lactones with known anticancer activity.

In the laboratory of Chemistry of Natural Compounds, IOCCP-BAS, shoot cultures of the Balkan endemic ***Sideritis scardica*** Griseb., **Lamiaceae** are being maintained. A comparative analysis of enriched fractions of a commercial sample and *in vitro* cultures material has shown that fractions containing predominantly flavonoid-diglycoside compounds express a marked cytotoxic effect against MCF-7 cancer cell line, while fractions containing phenylethanoids do not affect cancer cells division. Preliminary results on the biotechnological optimization of shoot cultures of the plant have led to obtaining *in vitro* systems for the differential stimulation of compounds of each of these chemical classes. The complete implementation of the experiment will lead to the in-depth understanding of the factors related to their biogenesis and will have practical value for obtaining extractable biomass with defined phytotherapeutic potential of this plant.

3.2. Work on genetically non-transformed root cultures of the cultivated plants

In the laboratory of Chemistry of Natural Compounds, IOCCP-BAS, genetically non-transformed root lines of ***Inula britannica*** are being cultivated in liquid cultures. Preliminary research indicates that light treatment stimulates phenolic acids production and the auxin type also has effect on the production of these compounds. Roots, grown in PGR supplemented liquid medium have significantly higher phenolic productivity as compared with the roots of the sterile whole plant grown in agar medium.

Implementation of the planned experimental work will allow for better understanding of biosynthetic processes occurring in the root tissue within the whole plant organism by

means of their comparison with roots cultures which are grown without the presence of aerial parts.

3.3. Work on the non-differentiated lines of plants grown in liquid media

In the laboratory of Chemistry of Natural Compounds, IOCCP-BAS, non-differentiated lines of cell aggregates of *Artemisia alba* are being grown in liquid media. Preliminary research shows that light treatment and PGR affect the production of phenolic acids, as well as coumarins scopoletin and fraxidin-8-glucoside, as well the production of stress hormones salicylic, abscisic and jasmonic acid. In addition, the *in vitro* stress levels of the initial explants of which cell cultures have been started affect the physiological status and phenolicss production.

Implementation of the planned experiments will allow for the better understanding of factors affecting coumarin *in vitro* production and will allow for obtaining an *in vitro* model for their controlled delivery.

4. Bibliography

4.1. Bibliography of literature sources of other authors used in the

Introduction

- Akkermans A.D.L., W. Roelofsen, J. Blom, K. Hussdanell, and R. Harkink., *Can. J. Bot.*, **61**, 1983, 2793–2800.
- Baker D. D., B. C. Mullin, In: Biological nitrogen fixation (ed. G. Stacey, R. H. Burris, and H. J. Evans), New York, Chapman & Hall, 1992, 259–292.
- Bulgarian Law for Medicinal Plants (2017) <https://lex.bg/laws/ldoc/2134916096>
- Burk, D.R., Senechal-Willis, P., Lopez, L.C., Hogue, B.G., Daskalova, S.M., 2009. Suppression of lipopolysaccharide-induced inflammatory responses in RAW 264.7 murine macrophages by aqueous extract of *Clinopodium vulgare* L. (Lamiaceae). *Journal of Ethnopharmacology*, **126**: 397–405
- CBD. Fourth National Report. Bulgaria. 2005-2008. Republic of Bulgaria Ministry of Environment And Water. <https://www.cbd.int/doc/world/bg/bg-nr-04-en.pdf>
- Dias MI, Sousa MJ, Alves RC, Ferreira ICFR (2016) Exploring plant tissue culture to improve the production of phenolic compounds: A review. *Industrial Crops and Products* 82: 9–22
- Dzhambazov, B., Daskalova, S., Montevea, A., Popov, N., 2002. *In vitro* screening for antitumour activity of *Clinopodium vulgare* L. (Lamiaceae) extracts. *Biol Pharm Bull*, **25**:499-504
- Gao X., M. Ohlander, N. Jeppsson, L. Bjork, V. Trajkovski, J. Of Agric. And Food Chemistry, **48**, 2000,1485–90.
- Holobiuc I, Blindu R (2006-2007) *In vitro* culture introduction for *ex situ* conservation of some rare plant species Rom J Biol - Plant Biol 51-52: 13 - 23, Bucharest
- IPCC. Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report (AR4), published in 2007
- Kumar P., S. Namita and H.C. Goel, *Mol Cell Biochem*, **238**, 2002, 1-2, 1-9.
- Li T.S.C, L.C.H. Wang. In: Functional foods, biochemical & processing aspects (ed. G. Mazza), Technomic Publ. Co. Inc., Lancaster, PA, 1998, p. 329–56.
- Lombardino JG (2000) A brief history of Pfizer Central Research. *Bull. Hist. Chem.*25: 10-15.
- Loyola-Vargas, V.M., Vázquez-Flota, F. (Eds.), 2006. *Plant Cell Culture Protocols*, vol.318. Humana Press, Totowa, New Jersey.
- Ochoa-Villarreal M, Howat S, Hong SM, Jang MO, Jin YW, Lee EK, Loake GJ (2016) Plant cell culture strategies for the production of natural products. *BMB Rep.* 49: 149-158
- Petrova, A., Vladimirov, V. 2007. Recent (1994–2004) taxonomic studies on the Bulgarian flora. – *Bocconea*, 21: 7-25.
- Petrova, A., Vladimirov, V. (eds). 2009. Red List of Bulgarian vascular plants. *Phytologia Balcanica* 15 (1): 63 – 94, Sofia.
- Radulović N, Blagojević P (2010) Volatile profiles of *Artemisia alba* Turra from contrasting serpentine and calcareous habitats. *Natural Product Communications*, 5: 1117-1122
- Ronse A, De Pooter HL. (1990) Essential oil production by Belgian *Artemisia alba* (Turra) before and after micropropagation. *Journal of Essential Oil Research*, **2**, 237-242;
- Routian JB, Nickell LG (1956) US Patent 2 747 334

Roussi A., Ann. Bot. Fenn., **8**, 1971, 177–227.

Stojanovic G, Palic R, Mitrovic J. (2000) Chemical composition and antimicrobial activity of the essential oil of *Artemisia lobelii* All. *Journal of Essential Oil Research*, **12**, 621-624.

Suleyman H., L.O. Demirezer, M.E. Buyukokuroglu, M.F. Akcay, A. Gepdiremen, Z.N. Banoglu, F. Gocer , *Phytotherapy Research*, **15**, 2001, 625–7.

4.2. Bibliography of “B” publications of the candidate included in the habilitation contributions

B-Q1

1) Danova, K.; Todorova, M.; Trendafilova, A.; Evstatieva, L. *Natural Product Communications*, 2012, 7, 1075 – 1076 (**Cytokinin and Auxin Effect on the Terpenoid Profile of the Essential Oil and Morphological Characteristics of Shoot Cultures of *Artemisia alba***)

2) Danova, K.; Motyka, V.; Todorova, M.; Trendafilova, A.; Krumova, S.; Dobrev, P.; Andreeva, T.; Oreshkova, T.; Taneva, S.; Evstatieva, L. *Journal of Plant Growth Regulation*. 2018, 37, 403–418 (**Effect of Cytokinin and Auxin Treatments on Morphogenesis, Terpenoid Biosynthesis, Photosystem Structural Organization, and Endogenous Isoprenoid Cytokinin Profile in *Artemisia alba* Turra In Vitro**)

B-Q2

1) Petrova, A.; Danova, K.; Kapchina-Toteva, V. *Comptes rendus de l'Académie bulgare des Sciences*, 2008,61, 363–370. (**Ecological Evaluation and Conservational Value for Bulgaria of *Hippophae Rhamnoides* L. Total Flavonoids Determination and Experiments on *In Vitro* Culture Induction**)

B-Q3

1) Petrova,N.; Koleva,P.; Velikova,V.; Tsonev,T.; Andreeva,T.; Taneva,S.; Krumova,S.; Danova,K. *International Journal Bioautomation*, 2018, 22, 73-82. (**Relations between Photosynthetic Performance and Polyphenolics Productivity of *Artemisia alba* Turra in *in vitro* Tissue Cultures**)

2) Krumova, S.; Motyka, V.; Dobrev, P.; Todorova, M.; Trendafilova, A.; Evstatieva, L.; Danova, K. *Bulgarian Journal of Agricultural Science*, 2013, 19, 26-30. (**Terpenoid Profile of *Artemisia alba* is Related to Endogenous Cytokinins *In Vitro***)

3) Mehandzhiyski, A.; Batovska, D.; Dimitrov, D.; Evstatieva, L.; Danova, K. *Bulgarian Journal of Agricultural Sciences*, 2013, 19,31-34. (**Nitric Oxide-Scavenging Activity of *In Vitro* Cultured Balkan Medicinal and Aromatic Plants**)

4) Treneva, G.; Markovska, Y.; Wolfram, E.; Danova, K. *Bulgarian Journal of Agricultural Sciences*, 2014, 20, 46-50. (**Effect of Plant Growth Regulators on Growth Patterns and Enzymatic Antioxidant Activities in *Hypericum calycinum* Shoot Cultures**)

4.3. Bibliography of “T” publications of the candidate in addition to the habilitation contributions

Г-Q1

- 1) Danova, K.; Nikolova-Damianova, B.; Denev, R.; Dimitrov, D. Plant Cell Tissue and Organ Culture. 2012, 110, 383–393. **(Influence of vitamins on polyphenolic content, morphological development, and stress response in shoot cultures of *Hypericum* spp)**
- 2) Danova, K.; Nikolova-Damianova, B.; Denev, R.; Markovska, Y. Plant Growth Regulation. 2012, 68, 447–457. **(Impact of pre-culture on short- and long-term *in vitro* recovery of the biosynthetic potential and enzymatic and non-enzymatic antioxidant defense of *Hypericum rumeliacum* Boiss. after cryostorage)**
- 3) Todorova, M.; Trendafilova, A.; Danova, K.; Simmons, L.; Wolfram, E.; Meier, B.; Riedl, R.; Evstatieva, L. Phytochemistry, 2015, 110, 140-149. **(Highly oxygenated sesquiterpenes in *Artemisia alba* Turra)**

Г-Q2

- 1) Todorova, M.; Trendafilova, A.; Ivanova, V.; Danova, K.; Dimitrov, D. Natural Product Research, 2017, 31, 1693-1696. **(Essential oil composition of *Inula britannica* L. from Bulgaria)**
- 2) Ivanova, V.; Trendafilova, A.; Todorova, M.; Danova, K.; Dimitrov, D. Natural Product Communications, 2017, 12, 153-154. **(Phytochemical Profile of *Inula britannica* from Bulgaria)**
- 3) Trendafilova, A.; Todorova, M.; Genova, V.; Peter, S.; Wolfram, E.; Danova, K.; Evstatieva, L. Chemistry & Biodiversity, 2018, 15. **(Phenolic Profile of *Artemisia alba* Turra)**
- 4) Aneva, I.; Zhelev P.; Kozuharova E.; Danova, K.; Nabavi S.F.; Behzad, S. DARU Journal of Pharmaceutical Science. 2019, <https://doi.org/10.1007/s40199-019-00261-8>. **(Genus *Sideritis*, section *Empedoclia* in southeastern Europe and Turkey – studies in ethnopharmacology and recent progress of biological activities)**

Г-Q3

- 1) Todorova, M.; Trendafilova, A.; Danova, K.; Dimitrov, D. Biochemical Systematics and Ecology, 2011, 39, 4-6. **(Phytochemical study of *Anthemis rumelica* (Velen.) Stoj. & Acht.)**

Г-Q4

- 1) Mártonfióvá, L.; Danova, K.; Toteva, V.K.; Čellárová, E. Thaiszia - J. Bot., 2, 2014, 24, 143-150. **(Karyotype analysis of *Hypericum rumeliacum* Boiss.)**
- 2) Amer, H.M. ; El-Gohary, A.E.; Hendawy, S.F.; Hussein, M.S., Danova, K. Bioscience Research, 2019, 6, 561-572. **(Improvement of growth parameters and essential oil productivity of *Anthriscus cerefolium* L. by planting distances and fertilization treatments)**
- 3) Danova, K.; Ionkova, I.; Vasilev, N.; Ninov, St.; Troiantcheva, B. Pharmacia, 1-2 vol. LII, 2005, 56-59. **(Influence of the composition of nutrition media on the production of aryltetralin lignans in *Linum tauricum* ssp. *tauricum* (WILLD) Petrova cellular and tissue cultures)**

Book chapters

Г-Book Ch

- 1) Danova, K. **Biotechnological utilization of the indigenous biosynthetic capacity of medicinal and aromatic plants. Experience in the genera *Hypericum*, *Pulsatilla* and essential oil bearing *Artemisia alba* characteristic for the Balkan region**; Book chapter for Series Recent Progress in Medicinal Plants; Vol.39: Biotechnology and Genetic Engineering II, 2014, 355–392 pp., Series ISBN: 0-9656038-5-7, Vol ISBN: 1-933699-99-X, Studium Press LLC, USA
- 2) Danova, K. (2015) **Potential of the Balkan Flora as a Source of Prospective *Hypericum* Genotypes for the Conventional and Biotechnological Delivery of Phytopharmaceuticals. Chapter 2 in: *Hypericum: Botanical Sources, Medical Properties and Health Effects*. Howard R. Davis (Ed), Series Plant Science Research and Practices, Nova Science Publishers, USA, ISBN: 978-1-63482-701-0, pages 19-52**
- 3) Danova, K. **Roles of Developmental Patterns and Morphogenesis in the Secondary Metabolite Production of Conventionally and Biotechnologically Cultivated Medicinal and Aromatic Plants. Chapter 1 in: *Recent Advances in Plant Research***; Editors: Beatrice Welch and Micheal Wilkerson; Series: Plant Science Research and Practices. Nova Science Publishers, USA, ISBN: 978-1-53614-170-2, 2018

4.4. Other publications of the candidate

1. Danova K., Y. Markovska, D. Dimitrov, V. Kapchina-Toteva (2007) In vitro culture initiation and phenol and flavonoid determination of some medicinal plants, endemic to the Balkan flora, Proceedings book of International Scientific Conference Stara Zagora, June 7-8, 2007, vol. 1 “Plant breeding”, pp 222 - 229.
2. Danova K., Damianova P., Kapchina-Toteva V. (2007) Utilization of the methods of in vitro propagation for resource purposes in medicinal plants breeding. In vitro cultivation of some *Hypericum* species. Journal of mountain agriculture 10(6): 1074-1089.
3. Danova K., Yordanova Zh., Markovska Yu, Čellarova E, Kapchina-Toteva V (2007) Propagation in vitro of *Orthosiphon stamineus*, proceedings book of International Scientific Conference Stara Zagora, JUNE 7-8, 2007, pp 406-411.
4. Gorgorov R, Danova K., Dimitrov D., Markovska Y., Kapchina-Toteva V. (2009) Micropropagation and some secondary metabolites of *Lamium album*, Proceedings Book of International Scientific Conference, Stara Zagora 5-6 June, 131-135.
5. Danova K, Kapchina-Toteva V. Cryopreservation – a new method for conservation of *Hypericum rumeliacum* Boiss. (2009) Proceedings of the International Scientific Conference (June, 2009, Stara Zagora, Bulgaria), vol. 3 “Medical Biol. Studies”, pp 90-95.
6. Danova K, Urbanová M, Skyba M, Čelárová E, Stefanova M, Koleva D, Kapchina-Toteva V (2009) Impact of cryopreservation on biochemical parameters of in vitro cultured *Hypericum rumeliacum* Boiss. (2009) Proceedings of International Symposium “New Research in Biotechnology” (19-20 Nov, Bucharest), pp 78-85.

7. Danova K, Čellárová E, Kapchina-Toteva V. (2010) Impact of growth regulators on in vitro regeneration of *Hypericum rumeliacum* Boiss., *Journal of Environmental Prot and Ecol*, 11: 1285-1292. **IF = 0.16**
8. Danova K., Čellárová E., Macková A., Daxnerová Z, Kapchina-Toteva V, (2010) In vitro culture of *Hypericum rumeliacum* Boiss. and production of phenolics and flavonoids. *In Vitro Cellular and Developmental Biology – Plant* 46: 422-429 **IF = 1.48**
9. Danova K (2010) Production of polyphenolic compounds in shoot cultures of *Hypericum* species characteristic for The Balkan Flora. *Botanica Serbica*, 34(1): 29-36.
10. Danova K, Bertoli A, Pistelli Laura, Dimitrov D, Pistelli Lu (2009) In vitro culture of Balkan endemic and rare *Pulsatilla* species for conservational purposes and secondary metabolites production. *Botanica Serbica*, 33(2): 157-162.
11. Dimitrova M, Dragolova D, Lyudmilov V, Danova K, Dimitrov D, Kapchina-Toteva V (2009) Micropropagation of *Leonurus cardiaca* – influence of auxins and cytokinins. *General and Applied Plant Physiology*, Volume 35 (3–4), pp. 146–152
12. Miladinova K., Georgieva T., Ivanova K., Geneva M., Danova K., Markovska Y. (2012) Ex vitro growth and antioxidative responses of two *Pawlownia* clones to Zn excess. *Proceedings book, International conference, Ecology- interdisciplinary science and practice, Sofia 25-26 Oct. 2012*, pp.526-530.
13. Miladinova K., Georgieva T., Ivanova K., Geneva M., Danova K., Markovska Y. (2012) Cadmium and lead effects on ex vitro growth and antioxidative response of two *Paulownia* clones. *Proceedings book, International conference, Ecology- interdisciplinary science and practice, Sofia 25-26 Oct. 2012*, pp. 520-525.
14. Raynova Y, Markovska Y, Idakieva K, Wolfram E, Danova K. (2014) Relations between enzymatic and non-enzymatic antioxidant defence involved in polyphenolics production of *Artemisia alba* in vitro, *proceedings book, Seminar of Ecology, Union of Scientist, Bulgaria, Sofia 24-25 April 2013*, pp 134-138
15. Treneva, G.; Markovska, Y.; Wolfram, E.; Danova, K. (2014) Effect of plant growth regulators on growth patterns and enzymatic antioxidant activities in *Hypericum calycinum* shoot cultures, *Bulg J Agr Sci* (2014) 20 (1) 2014, 000–000, P-ISSN 1310-0351 – **IF = 0.3**
16. Todorova M., Trendafilova A., Krumova S., Idakieva K., Genova V., Markovska Y., Raynova Y., Evstatieva L., Wolfram E., Danova K. (2015) Interdisciplinary interaction for the biotechnological development of Balkan medicinal plant species, *Proceedings book of the Seminar of Ecology 2014*, 24-25 April, Union of Scientists, Bulgaria, Section Biology, pp 95-98.
17. Raynova Y, Idakieva K. Markovska Y, Wolfram-Schilling E., Danova K. (2016) Protein profiling and antioxidant enzyme activity of *Artemisia alba* in vitro cultures, *Proceedings book of Seminar of Ecology with International participation*, 23-24 April 2015, Sofia, Bulgaria pp160-162.
18. Krumova S., Andreeva T., Oreshkova Ts., Motyka V., Dobrev P., Todorova M., Trendafilova A., Petrova N., Taneva St., Danova K. (2016) Do endogenous cytokinins regulate terpenoid biogenesis and thylakoid morphogenesis in *Artemisia alba* in vitro model system? (2016) *Proceedings book of Seminar of Ecology with International participation*, 23-24 April 2015, Sofia, Bulgaria pp 163-165

19. Azaiez S., Slimene-Debez I., Limam F., Tounsi M., Hamami M., Mliki A., Bouamama B., Jebara M., Borji M., Danova K (2016) Screening of the antimicrobial activity of *in vitro* cultivated medicinal plants originating from Bulgaria and Tunisia (2016) Proceedings book of Seminar of Ecology with International participation, 23-24 April 2015, Sofia, Bulgaria pp 165-167
20. Hendawy SF, El-Gendy AG, El Gohary AE, Hussein MS, Danova K, Omer EA (2015) Evaluation of biomass formation, essential oil yield and composition of four different *Matricaria recutita* L. cultivars grown in Egypt. World Journal of Pharmaceutical Sciences, ISSN (Print): 2321-3310; ISSN (Online): 2321-3086 **IF = 0.453**
21. Koleva P., Wolfram E., Pedrussio S., Raynova Y., Evstatieva L., Danova K. (2015) In vitro culture development and polyphenolics production of *Artemisia alba* Turra. J. Bio Sci. Biotechnol. 2015, SE/ONLINE: 131-136
22. Danova K., Koleva P., Aneva I., Evstatieva L (2017) Multiplication and polyphenolics production of *Sideritis scardica* through different tissue culture approaches. Proceedings book of Seminar of Ecology with International participation, 21-22 April 2016, Sofia, Bulgaria pp 100-104
23. **Kalina Danova**, Petre I. Dobrev, **Antoaneta Trendafilova**, **Milka Todorova**, Vaclav Motyka. Modification of morphogenetic patterns in tissue cultures of *Artemisia alba turra* as a key for secondary metabolites targeting. SGEM Geo Conferences, **2018**, ISBN:978-619-7408-3, ISSN:1314-2704, DOI:<https://doi.org/10.5593/sgem2018v/6.4/s08.028>, 219-226
24. **Petya Koleva**, Elena Stoyanova, **Kalina Alipieva**, Ina Aneva, Ljuba Evstatieva, **Kalina Danova**. Cytotoxic activity of *Sideritis scardica* extracts and fractions on human breast adenocarcinoma cell line MCF7. Proceedings Book of the 10th Anniversary SEMINAR OF ECOLOGY - 2017, ФАРАГО, **2018**, ISBN:979-853-476-132-4, 124-126
25. Kalina Danova, Vaclav Motyka, Petre Dobrev (2019) Effects of *in vitro* morphogenesis and developmental patterns of *Artemisia alba* Turra on polyphenolics production and endogenous stress hormones. Proceedings Book of the 11th SEMINAR OF ECOLOGY - 2018, Publisher: Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, **2018**, ISBN: 978-954-9746-45-7