# Final

# R E P O R T

**ON**

**THE RESEARCH CARRIED OUT**

**IN**

**PROJECT КП-06-Н26-6/13.12.2018**

**FUNDED BY THE BULGARIAN SCIENTIFIC RESEARCH FUND**

**WITH**

**THEME: " „ RERODUTIVE POTENTIAL, METABOLIC AND GENETIC PROFILE, IN IN SITU AND EX SITU CONDITIONS, OF MEDICINAL PLANTSSPECIES FROM THE BULGARIAN FLORA WITH RESSOURCE DEFICIT - SCIENTIFIC BASE FOR THEIR CULTIVATION"**

**DECEMBER 2018 – FEBRUARY 2023**

PROJECT MANAGER: Assoc. Prof. Elina Petrova Yankova-Tsvetkova, PhD /IBER-BAS/

**S O F I A**

**2023**

**C O N T E N T S**

## 1. Absract

**Objective:** The main strategic objective of this project is cultivation with a view to providing raw material and protecting the natural populations of four valuable medicinal plants with a resource deficit and conservation value for the Bulgarian flora, namely: *Alkanna tinctoria, Helichrysum arenarium, Primula veris, Arctostaphylos uva- ursi*.

**The operational objectives of the project are:** monitoring of the natural populations of the target species; establishing their state through their main characteristics: size, abundance, operating stocks, etc.; revealing the type of reproduction, the realization of their reproductive potential under specific environmental conditions (including stressful ones); determination of the content of biologically active substances /BAS/, genetic and metabolic profile; establishing the optimal conditions for effective micropropagation; creation and maintenance of *ex situ* collections for storage of valuable genetic material needed for future activities related to conservation of target species through cultivation.

**Research strategy:** To achieve these goals, a complex of studies is applied, which include: Monitoring of populations based on the "Methodology for monitoring of higher plants", "Methodology for assessing the condition of higher plants" of the Executive Agency for the Environment (EAES) ; Embryological studies to determine the type of reproduction and the reproductive potential of the target species; The development of protocols for *in vitro* propagation and acclimatization in natural conditions of the target species by applying different biotechnological approaches: clonal micropropagation, somatic embryogenesis, hairy root cultures, hydroponic cultivation; Phytochemical studies to determine the content of basic BAS by chromatographic /GC-MS, TLC, HPLC/ and spectrophotometric methods; Genetic analyzes using DNA markers and Liquid cytometry to assess genetic diversity and genome size of target species; Creation of *ex situ* plant collections from natural habitats and from *in vitro* and hydroponically propagated plants.

**Expected results:** On the basis of this complex study, fundamental scientific data will be obtained for the selected little-studied medicinal species of conservation importance for the Bulgarian flora. They will complement the biological characteristics of the target species and create a basis for their future breeding in culture.

## 2. Scientific team

Assoc. Prof. Elina Petrova Yankova-Tsvetkova, PhD – project manager

Organizations/team members:

**Institute of Biodiversity and Ecosystem Research - BAS - Applying organization**

Prof. PhD Strahil Hristov Berkov, PhD

Assoc. Prof. Marina Ivanova Stanilova, PhD

Assoc. Prof. Petka Dimitrova Yurukova-Grancharova, PhD

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Assoc. Prof. Vladimir Dimitrov Vladimirov

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Biologist Rozalia Bojidarova Nikolova

Agronomist Tatyana Tihomirova Stefanova

Chemist Marina Nikolova Dimitrova

**Agricultural University, Plovdiv – Partner organization**

Assoc. Prof. Ivanka Bozhkova Semerdjieva, PhD

Prof. PhD Valtcho ZheJeljazkov, PhD

## 3. Description

The object of research in the current project are the following medicinal plants: *Alkanna tinctoria, Helichrysum arenarium, Primula veris and Arctostaphylos uva-ursi*. When selecting the target species, it was taken into account that they are valuable, marketed medicinal plants, with conservation significance for the Bulgarian flora, under a special regime of use, according to the annual Order of the Minister of the Environment and Waters, on the special regime of conservation and use of medicinal plants. The selected target species are poorly or not at all studied in terms of their reproductive potential and their possibilities for *ex situ* propagation. Elucidation of reproductive possibilities is a key point in the conservation of their natural populations. The only way for the sustainable use and protection of these resources for the Bulgarian flora is through the development of approaches for *ex situ* cultivation of the target species, which requires a complex research approach. In this regard, in the present project it is proposed to conduct interdisciplinary research, which has not been done with the target species so far.

Brief description of the species proposed for research:

***Alkanna tinctoria*** **(L.) Tausch (alkanet) (Boraginaceae)** is a species included in the Red Data Book of the Republic of Bulgaria (2015), in the Red List of Higher Plants (Petrova & Vladimirov 2009) with the category „endangered species“ and in Appendix 3 of the Biodiversity Act (BDA 2002) for species forbidden for collection from their natural habitats.

*Distribution in Bulgaria*: There are several small populations in the plains and foothills of southern Bulgaria and one in the Danube plain. The plant reproduces by seeds and is found on stony and sandy places from 100 to 600 m above sea level.

***Primula veris*** **L. (clowslip) (family Primulaceae)** is a protected species, according to the Biodiversity Act. The species is included in Appendix 4 of the Act, and in the List of medicinal plant species under a special regime of protection and use, on the basis of the Act of Medicinal Plants (Art. 10, paras. 1, 2 and 3). A species with annual amounts of herbs for economic purposes determined by the Ministry of the Environment and Water /MEW/ from their natural habitats, outside the territories of national parks. The aerial parts and roots of the primrose are allowed to be collected from the natural deposits, and annually MEW sets quotas for their collection.

*Distribution in Bulgaria*: Mainly in the oak belt throughout the country, but most often the populations of the species are of low abundance and small area and uneven distribution of the plants. Larger populations are actively used for the collection of flowering stems and roots for economic purposes. The species reproduces by seeds, forming multi-colored seeds, and vegetatively by root shoots. As a result of the annual collection of plant materials for pharmaceutical purposes, some of the economically significant deposits of primrose have been depleted.

***Arctostaphylos uva-ursi (L.) Sprenq. (Bear grape) (family Ericaceae)*** is a protected species according to the Biodiversity Act, is included in Appendix 4 of the Act, and in the List of species of medicinal plants under a special regime of protection and use on the basis of the Act of Medicinal Plants (Art. 10, paras. 1, 2 and 3). The species is forbidden to be collected

for commercial purposes from the natural habitats throughout the country. It is included in the list of medicinal plants of the Annexes to the Convention on International Trade with Endangered Species of Wild Fauna and Flora.
*Distribution in Bulgaria:* In almost all the mountains, at 1000-2500 m of altitude. Due to the active collection of the plant in the past and the climatic changes, big part of its populations has a reduced area.

***Helichrysum arenarium* (L.) Moench (everlasting) (Asteraceae)** is a protected species according to the Biodiversity Act /2007/, is included in Appendix 4 of the Act, and in the List of species of medicinal plants under a special regime of protection and use on the basis of the Act of Medicinal Plants (Art. 10, paras. 1, 2 and 3). ***The species is forbidden to be collected for commercial purposes from the natural habitats throughout the country.***

*Distribution in Bulgaria:* It grows in sandy places, scattered by the sea, in North-Eastern Bulgaria, Eastern Stara Planina and the Upper Thracian Plain from 0 to 400 m above sea level. It reproduces by seed and vegetatively.

## 4. Results

Due to the complex type of the study, the conducted studies are grouped into separate work packages:

### *4.1. Work package 1. In situ and ex situ studies and collection of source material:*

WP1 is composed of scientists from the two partnering scientific organizations who are experts in the field of plant diversity, monitoring and resource assessment of conservationally important, rare, medicinal and aromatic plants, and creation of *ex situ* collections of them: Assoc. Prof. d-r. A. Vitkova, PhD, Assoc. Prof. d-r Stoyan Stoyanov, PhD /IBEI-BAN/, Assoc. Prof. d-r Ivanka Semerdzhieva, PhD, Prof. Valcho Zhelyazkov /Agrarian University Plovdiv/.

#### 4.1.1. Research methods and techniques

• Study of chorology of the selected species by collecting information on their natural habitats from the Bulgarian herbaria: Institute of Biodiversity and Ecosystem Research (SOM), Biological Faculty of the University of Sofia (SO), Agricultural University, Plovdiv (SOA), from literature sources and realizing field studies on the territory of the country.

• Localization of selected populations of the target species by route and stationary method and with the help of GPS devices.

• Study of the target species in model habitats by monitoring made according "Methodology for monitoring of vascular plants" and "Methodology for estimation the state of higher plants" from National monitoring system of biodiversity of the Executive Environment Agency (http://eea.government.bg/bg/nsmos).

• Observation of the ecological characteristics of natural habitats of the target species - altitude, exposure, slope, humidity, type of soil substrate, in order to be taken into account for their cultivation.

• Phenological observations on the target species according to the methodological instructions of Bideman (1974) in order to establish the growth and development levels.

• Collection of the initial plant material from the target species, according to their biological characteristics, in quantities agreed with the Ministry of Environment and Water /MEW/ and regulated on the basis of Authorization under the Biological Diversity Act (2002) for the purposes of research conducted by the other WPs.

• Attempts for *ex situ* seed and vegetative propagation according to Brouz (1987); seed quality and germination ability - according to Ovcharov (1976) and Bewley & al. (1985).

• Experimental cultivation of the studied species after Evstatieva & Stanev (2005) in the *ex situ* collections of IBER /Sofia and Vitosha/ and Agricultural University, Plovdiv.

***4.1.2.Tasks according to the work program of the project***

***1. Localization and description of populations of the target species and collection of material for the creation of ex situ collections of the target species and for scientific research on the other tasks of the project***

***2. Monitoring of natural populations of target species***

***3. Attempts to cultivate the target species at the experimental areas of IBEI, BAS and AU Plovdiv***

**4.1.3. Results**

**4.1.3.1. Horological reference**

A horological reference was made for the studied species based on the herbarium specimens in the herbariums - (SOM) IBEI, BAS; (SO) SU "Kl. Ohridski"; (SOA) Agricultural University - Plovdiv and literary sources, and the results are presented in the corresponding horological tables for each species studied.

**4.1.3.2. *In situ* and *ex situ* studies**

In order to obtain actual data on the distribution of the target species and assess the state of their populations in the period 2019-2022, a number of field studies were conducted, as a result of which the distribution of the species in the country was determined using the route and stationary method. Basic ecological and phytocenotic parameters of the populations of the studied species were evaluated, such as area, altitude, exposure, type of soil, humidification, type of plant communities with their participation, numbers and/or projective coverage, taking into account the floristic area, described unit, phenological phase, GPS coordinates of the habitat, habitat characteristic, population area and density, projective cover, presence of invasive species, threats, on the base of "Higher Plant Monitoring Methodology" (Gussev et al 2008). The data are entered in unified forms approved by the Ministry of Environment and Waterby. From representative populations of the studied species, live plants were transferred and seeds collected for the creation of *ex situ* collections and the cultivation of the four medicinal species in culture, on soil substrates and hydroponic systems. *Ex situ* collections of the target species were created in the experimental bases of IBEI-experimental field and greenhouse and AU-experimental field.

***Alkanna tinctoria* (L.) Tausch**

1. **Localization and description of the populations of the species**

During the horological reference on *Alkanna tinctoria*, 76 herbarium specimens collected in the period 1930-2010 from natural populations in the country were found, as well as data on the distribution of the species published in 6 literary sources in the period 1918-1995. The most are the herbarium specimens samples /50.77%/ collected from the Valley of Struma river floristic region. From the Eastern Rhodopes they are 16.92%, from the Danube plain – 9.23%, Thracian lowland 6.15% and from the South Pirin region – 3.08%.

As a result of the conducted field studies, the current distribution of *Alkanna tinctoria* in Bulgaria was established, namely: the Valley of the Struma River and South Pirin /17 populations were described/, South-Eastern Bulgaria (Eastern Rhodopes) /3 populations were

described/ and the Danube Plain /described 2 populations/. The Localizations in Thracian lowland, Tundzhan hilly plain, Strandzha. and some of those in the Danube Plain and South-Eastern Bulgaria, described in literary sources and the herbarium specimens have not been confirmed. Probably the reason for their disappearance is the change of habitat conditions as a result of their abandonment or their burning (observed in the population in the village of Archar, Danube plain). The location, spatial structure, size and age structure of the populations were established. A map of the actual distribution of the species on the territory of the country has been made /Fig.1/.

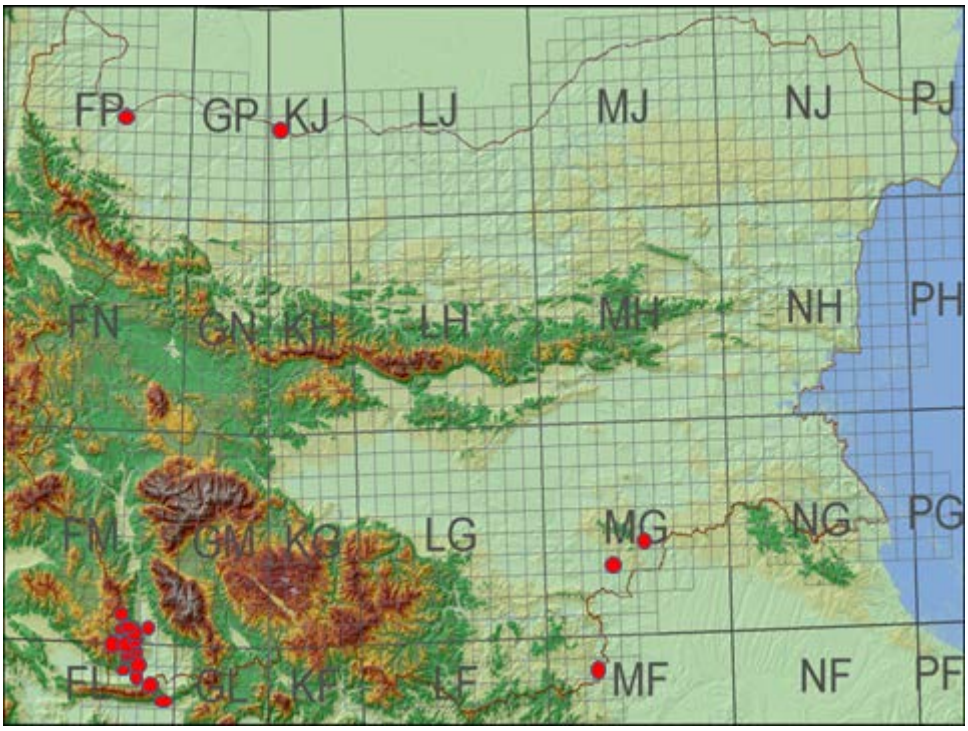


*Figure1. Distribution of A.tinctoria in Bulgaria*

**2. Monitoring**

The observed populations of *A. tinctoria* are fragmented, they occupy open, well-illuminated areas, with clay-sandy soil, and the species participates in the composition of grass and shrub-grass cenoses. The habitats of the described populations are dry, far from water sources, so moistening can only occur through precipitation. They are found at an altitude of 50 to 380 m above sea level, in grassy areas /Fig. 2/ and shrub communities /Fig. 3/. Differences are observed between the habitats of the populations in the different floristic regions: The studied populations in the Valley of Struma river grow on silicate or limestone stony slopes, in herbaceous communities, and a large number of them are composed of single plants or of not large number of small groups of individuals. They are in an unbalanced state /about 90% of the individuals in these populations are in the generative phase/. Influenced by a Mediterranean climate Populations in the Eastern Rhodopes grow on leptosol soils or calcareous rocks and are also influenced by a Mediterranean climate, flowering individuals predominate. Populations in the Danube Plain grow on inland sand dunes and are influenced

by a temperate-continental climate, and in these populations, the generative phase is predominant.

*Figure 2 Grassy communities*

*Figure 3 Shrub communities*

***Threats*:**

In most cases, the alkanet populations are located near settlements, arable lands and pastures, the reason for significant anthropogenic pressure on them, expressed in trampling, grazing of farm animals and erosion of the terrain. This anthropogenic pressure is more noticeable in the populations of the Valley of Strum river.

The monitoring carried out during the years of project implementation /2019-2022/ showed that the populations of the species maintain their size and are in good condition.

***Conclusion:***

As a result of the conducted field research, it was established that the populations of *A. tinctoria* in Bulgaria are localized in four floristic regions of the country: Valley of Struma river, South Pirin, Eastern Rhodopes and Danube Plain, and the majority localities are located in the Valley of Struma river, which is the main region of distribution of the species /the rest are represented by one population each/.

The low number of juvenile plants in all studied populations of *A. tinctoria* indicates that they are not in an equilibrium state and if parts of them vill be destroyed, they will have a hard time to recover.

**3.*Ex situ* studies**

An *ex situ* collection was created in the experimental field and in the greenhouse of IBEI and in the experimental field of AU Plovdiv, from individuals of *A tinctoria* transferred from the natural habitats and those grown hydroponically.

*Figure 4 In the greenhouse оранжерията*

*Figure 5 On the experimental field*

***Helichrysum arenarium***

## 1. Localization and description of the populations of the species

A horological reference was made for the distribution of the species in Bulgaria, which showed the existence of 27 herbarium specimens of H. arenarium in herbariums in the country, collected in the period 1895-2003 and 9 literary sources for the period 1901-2012. The largest is the list of herbarium specimens from the region of the Black Sea coast (north, south) – 51.85%, with the most frequently deposited herbarium spesimens from localities of the species around Varna, Pobitite Kamani, Beloslav. Another significant number of herbarium spesimens /37.03% / falls on the localities in North-Eastern Bulgaria (Provadia, Shumen, Kaspichan).

According to the literature data, the everlasting is distributed in 7 floristic regions of the country - North-Eastern Bulgaria, Black Sea coast, Pre-Balkan, Znepol region, Western border mountains, Danube plain and Thracian lowland. However, the review of the herbarium materials showed that these exist only from North-Eastern Bulgaria and the Black Sea coast.

As a result of the conducted field studies, the current distribution and localization of *H. arenarium* populations on the territory of Bulgaria was established /Fig.6/. The distribution of the species has been confirmed in the following floristic regions of the country: Northeast Bulgaria, Black Sea coast, Danube plain. 3 localities have been verified in North-Eastern Bulgaria and the Black Sea coast and a small one in the Danube Plain.

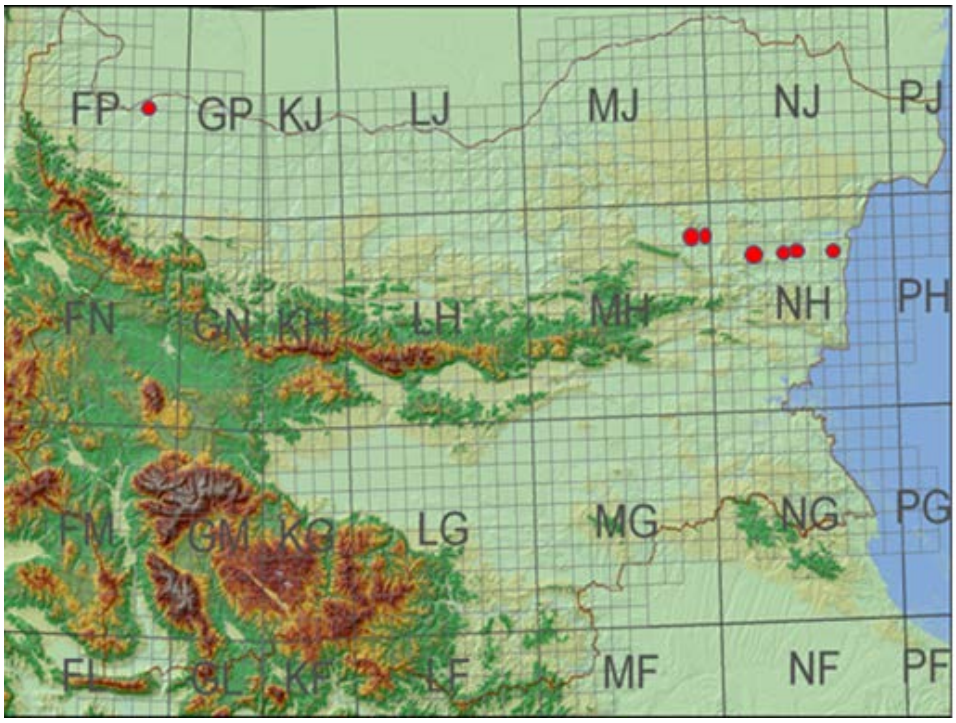


*Figure 6. Distribution of H. arenarium in Bulgaria*

## 2. Monitoring

*Helychrisum arenarium* participates in a habitat of European importance EUNIS E1.9B Sanding stone inland dunes. The habitat presents an outcrops of sands of Tertiary origin in the western part of the Varna tectonic depression on both sides of the Beloslav lakeTertiory origin in the western part of the Varna tectonic depression on both sides of the Lake of Beloslav /Fig.7/ They are part of grassy communities on calcareous sandy dry terrains located in plains and plateaus.

*Figure 7*

*Figure 8*

The vegetation is a complex of psamophytes, chasmophytes, forest and shrub cenoses. It was established that the populations of the species occupy leveled areas covered with sand, on a limestone bedrock, at an altitude of 90 to 380 m. and have an area of 00.5 to 5 ha. The

number of individuals in them varies between 0.1 and 0.5 numbers/m2. At the time of the research, the observed populations of *H. arenarium* are in good condition, with the exception of that of Ilchov bair - Shumen /Fig. 8 / which consists of no more than 100 individuals, which are smaller than those in the other studied populations.

The habitats of the described populations are dry, far from water sources, and moistening can only take place atmospherically /rainfall, dew/.

The monitoring carried out during the years of project implementation /2019-2022/ showed that the populations of the species maintain their size and are in good condition.

***Threats:***

Threats identified include soil erosion, development of competitive and invasive species. The populations are negatively affected by anthropogenic activities, such as the extraction of aggregates, trampling and the entry of invasive plant species, which suppress the development of *H. arenarium*. A large number of the populations reported in the literature were not found, due to the implementation of agricultural and construction activities in the indicated areas, which led to their destruction.

***Conclusion:***

Within the framework of the current research, the distribution of *H. arenarium* in Bulgaria has been established, mainly in six populations, in the northeastern part of the country, in a narrow strip between the cities of Shumen and Varna. This necessitates a review of the status of the species in the Bulgarian flora and its inclusion in the Biological Diversity Act as a rare species.

**3.*Ex situ* studies**

Създадена е *ex situ* колекция на опитното поле и в оранжерията на ИБЕИ и опитното поле на АУ Пловдив от индивиди на *H. arenarium* пренесени от естествените местообитания и такива размножени *in vitro* /Фиг. 9, 10/

An *ex situ* collection was created at the experimental field and in the greenhouse of IBEI and the experimental field of AU Plovdiv from individuals of *H. arenarium* transferred from the natural habitats and those propagated *in vitro* /Fig. 9, 10/

*Figure 9 In the greenhouse*

*Figure 10 On the experimental field*

***Primula veris***

**1. Localization and description of the populations of the species**

As a result of the horological reference, we found 76 herbarium specimens collected in the period 1888-1993 and 5 literary sources for the period 1909-1991 The distribution of the samples by floristic regions is: Stara planina (eastern, central, western) – 25%; Rhodopes (eastern, middle, western) – 38.16%; Western border mountains (Osogovo) – 6.58%; Sofia district (Vitosha), Znepol district - each 6.58%; Sredna Gora – 5.26% and Pirin Mt – 9.21%. Data were found on the distribution of the species in 14 of the 20 floristic regions in the country / Rhodopes, Stara Planina, Sredna Gora, Pirin Mt, Western Border Mountains, Sofia Region, Rila, Slavyanka, Pre-Balkan, Thracian Plain, Tundzhan Hilly Plain, Znepol Region , Belasitsa, Vitosha/. Horological data show that *Primula veris* in the country is represented by two subspecies - *Primula veris ssp. canescens*(Opiz)Hayek ex Lüdi in Hagi, distributed from 0 to 1000 m above sea level. and *Primula veris ssp. columne*(Ten) Lüdi in Hagi spread over 1000 m a.s.l.

**2. Monitoring**

During the implementation period of the project, populations of the species were studied in 3 floristic regions - Pirin Mt - above the village of Ilindentsi, Znepolski district - Golo Bardo above town of Pernik and Central Rhodopes - above the Martsiganitsa hut. The populations are located at 910 m.a.s.l./Znepolski district/, 1100 m.a.s.l./Pirin Mt/ and 1317

m.a.s.l./Rhodope Mts/ and occupy leveled terrain with a slope of 0-5$^{o}$. The population in the Pirin Mt is located on silicate bedrock and has an area of 0.2 ha and a low projective cover of the species of 0.2%. In the Znepol region, the population is located on a limestone bedrock with an area of 0.1ha and a projective species coverage of 0.4%, the population in the Rhodopes is located on limestone bedrock, with an area of about 0.5 ha and a projective species cover of about 0.7%

The monitoring carried out during the years of project implementation /2019-2022/ showed that the populations in Pirin Mt and Stara Planina have kept their size and are generally in good condition, while that of Golo Burdo /the smallest of the three populations/ has reduced its composition. The reason for this is probably the greatest proximity of this population to populated areas and its small number.

***Threats:***

The observations made on the studied populations of *P. veris* show that the more serious threats on their state are the anthropogenic impact and the natural low seed germination reported in most studies of the species.

***Conclusion:***

During the current study, it was found that the Bulgarian populations of *P. veris* are fragmented and strongly influenced by anthropogenic impact added to the naturally low seed germination.

**3.Ex situ studies**

For the purposes of the *ex situ* research, in 2019 we transferred living plants from the natural populations of the species, which we planted in the greenhouse of IBEI and on the experimental areas of IBEI and AU Plovdiv, with the aim of creating ex situ collections of the species /Fig. 11/. The IBEI collection was subsequently supplemented with plants grown from previously stimulated seeds.

*Figure 11. P. veris plants grown in the greenhouse and on the experimental field*

***Arctostaphylos uva ursi***

## 1. Localization and description of the populations of the species

During the horological reference, we found 66 herbarium specimens of bear grapes, collected in the period 1899-2007 and 6 literary sources from the period 1901-1961. The reference showed that the largest number of localities was reported for Vitosha - 24.24%; Stara Planina-28.79%; the remaining herbarium samples are: from Rhodope - 18.18%; Pirin - 15.15%, Slavyanka (Ali boot) – 12.12%

## 2. Monitoring

During the project implementation, five populations of the species were described and monitored: Vitosha /locality "Kamen del"/; Strara Planina /Kozyata stena/; Middle Rhodopes /Chairdere/; Pirin Mt /Betalovoto/ and Alibotush /Slavyanka.

For the studied populations of bear grapes, similar to the populations of most plant species, the group distribution is characteristic. It is determined by the local differences of the environment conditions, whereby individuals cluster in the more favorable conditions. The spatial structure of populations is not constant, but changes, depending on the occurring changes in the environment. The changes that occur are with adaptive character. The projective coverage of the bear grape in the described populations is in a very high percentage /almost 100%/

The monitoring carried out showed that all five populations are in a stable state in terms of their size and individual status.

***Threats:***

The conducted observations showed that the middle-aged groups predominate in the bearberry populations and there are almost no juvenile individuals. This is the reason why they are designated as "aging type". Anthropogenic impact is one of the main threats to the normal growth and development of the species. The increased interest in its healing properties is the reason for its excessive collection as an herb (Folium Uvae Ursi), which is accompanied by trampling of the localities and often with the uprooting of whole plants.

***Conclusion:***

Group distribution is characteristic for bearberry populations. *Arctostaphylos uva-ursi* (L.) Sprengel usually develops as a pioneer species on rocky and stony terrains with an optimum of development in the subalpine zone. In conditions of highly disturbed soil and plant cover, it can also participate in the construction of secondary communities. It develops well on rock outcrops and stones, where it initiates the formation of phytocenoses. This process is slow, and in the initial stages there are no other species in these communities, or if there are, they are very few. Therefore, the projective coverage of bear grapes often reaches 100%.

Anthropogenic impact is one of the main threats to the normal growth and development of bear grapes. It is necessary to take more measures to protect the species and restore its populations, in addition to the inclusion of its communities in the Red Book of

Bulgaria, Appendix No. 1 of the Law on Biological Diversity and within the borders of national and nature parks, as well as in protected areas of European ecological network Natura 2000. An important step is the monitoring observations and a timely reaction in the event of an emerging danger to the state of its habitats. Strict control and effective implementation of norms and regimes in protected areas is necessary.

**3.Ex situ studies**

An *ex situ* collection of vegetatively propagated rooted shoots from the mother plant was initiated in the IBEI greenhouse /Fig.12/

*Фигура 12. Ex situ growth rooted bearberry cuttings*

The problematic germination of the seeds and the slow growth of the plants under natural conditions also determined the slow growth of the rooted shoots under ex situ conditions /in the greenhouse/. Therefore, for more definitive results, more time is needed, which goes beyond the scope of the project.

An *ex situ* collection was also initiated of hydroponically rooted cuttings of Cranberry (*Vaccinium vitis-idaea* L.), used as a reference species in the present study, from the same family, also included in the List of species of medicinal plants under a special regime of conservation and use in Bulgaria, based on the Medicinal Plants Act (2000), participating together with Bearberry in the threatened habitat type 09F2 High Mountain Communities. Like Bearberry, it shows slow growth and development, but significantly more successful than Bearberry /Fig.13/.

*Figure 13. Hydroponically rooted cranberry cuttings grown in the greenhouse*

***Conclusion:***

The present study confirmed the difficulty in cultivating the bear grape and the need for more definitive and continuous research in order to establish the optimal conditions for the cultivation of this high-altitude plant inhabiting high-altitude terrains with specific ecological conditions.

### *4.2. Work package 2. Phytochemical characterization and metabolomics*

WP 2 is presented by Prof. d-r Strahil Berkov, PhD, Assoc. prof. d-r Milena Nikolova, PhD, and chemist Marina Dimitrova, who are specialized in the chemistry of alkaloids, analytical methods (GC-MS, HPLC-UV/MS, NMR) and their application in Biological active substances /BAS/ analyses, as well as in metabolomics, the study of BAS activity /surface flavonoids, antiradical activity/ and determination of the content, composition and dynamics of their accumulation in protected and valuable medicinal plants.

#### 4.2.1 Research methods and techniques

• Metabolomic analysis (lipids, sterols, amino acids, organic acids, mono-, di- and trisaccharides) were performed with gas chromatography-mass spectrometry (GC-MS). Metabolites were identified by comparison of their mass spectra and retention indices with those from mass spectral libraries (NIST 2008, GOLM) and literature data. Mass spectra were deconvolved with the AMDIS software package, and multivariate data analysis was performed with the Uncrambler X software package (Berkov & al. 2011). Metabolites that cannot be analyzed by GC-MS, such as saponins, were analyzed by high-performance liquid chromatography (HPLC).

• Antioxidant activity was evaluated by three experimental systems: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, phosphomolybdenum method and β -carotene/linolenic acid (Prieto & al. 1999; Kulisic & al. 2004; Marinova & Batchvarov 2011) .

#### 4.2.2. Tasks according to the work program of the project

***1 Preparation of the plant material collected from the natural populations of the studied species by treating it with methanol to obtain a total extract***

***2. Metabolic profiling of the extracts with GC-MS, TLC***

***3. Determination of the antioxidant potential of the studied samples***

#### 4.2.3. Results

***<u>Helichrysum arenarium</u>***

A comparative analysis of the metabolic profile of flower capitula from different populations of *H. arenarium* was carried out on the basis of acetone exudates obtained by extraction methods. Comparative analysis of the surface compounds isolated from flower capitula showed that the identified metabolites in the acetone exudates of *H. arenarium* were represented by phenolic acids, flavonoid aglycones, sterols and triterpenes, organic and fatty acids, sugars and sugar alcohols. The established metabolic profile of the Bulgarian populations, after a comparison with those described in the literature for the species, was determined as a new chemical type (hemotype) of *H. arenarium.*

A comparative analysis of the composition of the essential oil of the Bulgarian populations of the species with that of *H. italicum,* imposed on the market, was made. The results showed that the *H. arenarium* samples collected and analyzed in the present study had a specific essential oil profile that differed from the essential oil composition of *H. italicum*. Monoterpenes (α-pinene, sabinene) were found to be the predominant class of compounds in

*H. arenarium* essential oil, while *H. italicum* essential oil was dominated by sesquiterpenes (neryl acetate and β-hymahalene).

The surface exudate obtained from the flowers of *H arenarium* was evaluated by GC/MS for its inhibitory activity on the germination and initial root elongation of *Lolium perrene*, one of the most common crop weeds. In essence, this is the first report on the chemical composition of flower exudate of *H. arenarium*, the results of which are promising. The exudate solution at a concentration of 5 mg/mL was found to have a strong inhibitory effect, on both seed germination and initial root elongation of the resulting L. perrene seedlings. The flavonoid aglycon - naringenin, the monoterpenoid phenol - carvacrol, chlorogenic and 4-hydroxybenic acid have been identified as the main bioactive compounds of the exudate.

A comparative analysis of the antiradical capacity of leaves from *in vitro* grown and wild plants of *H.arenarium*, as well as flowers from wild and ex vitro adapted plants was made. Higher antiradical activity was found in plant organs from wild individuals.

***Alkanna tinctoria***

A comparative analysis of the metabolic profile of aboveground and underground parts of *Alkanna tinctoria* by GC/MS was performed.

Aerial parts of 11 populations of *A. tinctoria* were comparatively analyzed for metabolite content by GC/MS. Phenolic, organic and fatty acids, sterols, sugars and sugar alcohols were identified in methanolic extracts of the studied samples. Caffeic and 4-hydroxybenzoic acids have been identified as the main phenolic acids.

The total alkannin content in the hexane extracts of the roots collected from populations of the species from three different phytogeographical regions was spectrophotometrically determined: the Valley o Struma river, Danube Plain and Eastern Rhodopes. The amount of alkannin identified in the individual populations was compared with that of a commercial root product of *A. tinctoria*, where it was found that the alkannin content in some of the Valley of Struma river populations was close to and even exceeded in amount found in the commercial product, which is of foreign origin. These results outline the target populations from the Valley of Struma river as promising as initial populations for their cultivation as a source of alkannin.

A comparative study of the chemical profile of the aerial parts and roots, as well as of the total alkannin content in the roots of 11 populations of the species from different floristic regions of Bulgaria, was conducted. The aerial parts of *A. tinctoria* are poorly studied, not only in Bulgarian populations, but in the species as a whole. Methanolic extracts were analyzed using GC/MS. Phenolic, fatty and organic acids, sterols, polyols, fatty alcohols and sugars were identified. **The presence of ononitol in the aerial parts of the species, as well as phenolic acid, both in the aerial parts and in the roots, is reported for the first time.** The results show that the aerial parts of the plant are also promising for use as a source of valuable biologically active substances

***Primula veris***

The content of surface flavonoids in the Bulgarian populations of *P. veris* was analyzed by examining the metabolic profile of acetone exudates from the leaves of the studied populations of the species by GC/MS. Flavonoid aglycones have been identified as major bioactive compounds, and their amount reaches up to 50% of the total identified flavonoids.

Exudative compounds identified in *P. veris* leaves were tested as seed germination inhibitors and free radical scavengers. The composition of the exudate was analyzed by GC/MS. The seed germination test is carried out in petri dishes. Free radical scavenging activity was determined by DPPH assay. **This is the first report on the inhibitory activity on weed seed germination on cultivated plants of *P. veris* leaf exudates as well as their antiradical potential.** A complete inhibition of root growth and 89% inhibition of seed germination of *Lolium perenne* was found from an aqueous solution of exudate at a concentration of 5 mg/mL. The established antiradical activity is not high (IC50 >200 μg/mL).

***Arctostaphylos uva-ursi***

The bioactive compounds of the methanol extracts of leaf samples from three of the target populations of the species / Pirin, Vitosha and Middle Rhodope/ were identified by GC/MS and TLC. Total phenolic content was determined using the Folin-Ciocalteu reagent. Arbutin and quinic acid were found in the highest amounts. Gallic acid, catechin, 4-hydroxybenzoic acid, chlorogenic acid, triterpene (α- and β-amyrin, lupeol) vitamins and other primary and secondary metabolites were also detected by GC/MS in the investigated samples. Flavonoids (hyperoside, rutin, isoquercetin, quercetrin) were detected by TLC. Differences in the content of individual compounds were found between samples of different origins. However, no significant differences in total phenolic content were found between samples of different origins. The total phenolic content as well as the percentage of arbutin was also determined. Quantitative analysis showed that there were no significant differences between samples of different origins.

**Conclusion:**

- The metabolic profile of *Arctostaphylos uva-ursi* samples from three origins was determined. No significant differences in the profiles of the three origins were found. The highest content of arbutin was found in the sample from Vitosha, therefore it is defined as a promising source of origin for the cultivation of the species. The quantitative content of arbutin is influenced by habitat conditions

- The metabolic profile of leaf exudates of individuals from the natural population of *Primula veris* was analyzed and compared with that of individuals cultivated in the greenhouse after stimulation of seed germination. No differences were found. Flavonoid aglycones have been identified as major components of the exudate.

- The antioxidant activity of extracts from different organs of the target species *Arctostaphylos uva-ursi* (leaves), *Primula veris* (roots and leaves), *Alkanna tinctoria* (roots and aerial part), *Helichrysum arenarium* (flowers) was determined. Bearberry leaves have the highest radical scavenging activity.

- The metabolic profile and antioxidant potential of wild and *in vitro* propagated and adapted individuals of *Helichrysum arenarium* were comparatively analyzed. The extracts from the *in vitro* propagated individuals showed a lower antiradical activity. No differences were found in the metabolic profiles of wild and *in vitro* growth individuals.

- A new chemical type (hemotype) of *H. arenarium* was established in Bulgaria.

- The examined samples from the aereal and underground parts of *A. tinctoria* showed that they have a similar qualitative metabolic composition, with individual components varying in their quantitative representation between different origins. Myo-inositol, sucrose and fructose isomers are major components present in the highest amount in the methanolic extracts. Phenolic acids are better represented in the aerial parts. Underground sterols are higher, and the wild populations have higher sterol content than the commercial product.

- Crop weed inhibitory activity of *Primula veris* leaf exudates and *Helichrysum arenarium* flower capitula on crop weed seeds was found.

- The presence of ononitol was found in the aerial parts of *A. tinctoria*, which were also determined to be promising for use as a source of valuable biologically active substances.

**4.3.** ***Work package 3. Embryological studies***

WP 3 is represented by Assoc.prof. d-r Elina Yankova-Tsvetkova, PhD, Assoc.prof. d-r Petka Yurukova-Grancharova, PhD, young scientist/biologist Rozaliya Nikolova and biologist Tatiana Stefanova - representatives of the only scientific unit in Bulgaria, which conducts research in the field of the evolutionary, ecological and experimental embryology of wild species of Bulgarian flora, with a priority focus of research on the reproductive potential of rare, endangered and medicinal plant species.

**4.3.1**. **Research methods and techniques**

• Comparative-embryological studies based on the permanent microscopic preparations prepared according to the classic paraffin method, to establish the peculiarities of the structures and processes in the male and female generative sphere;

• Determination of pollen viability by Acetocarmine treatment (Singh 2003) and direct counting of fertile pollen using a light microscope (Heslop-Harrison 1992);

• Determination of seed (embryo) viability with the Tetrazole test (Peters 2000).

• Laboratory seed germination testing.

**4.3.2. Tasks according to the work program of the project**

***1. Embryological study. Establishing the characteristics of the male and female generative sphere and revealing the biology of reproduction***

***2. Evaluation of reproductive potential by establishing the degree of viability of pollen and seeds /in %/***

**4.3.3. Results**

Ten populations of *Alkanna tinctoria* (eight from the region of the Valley of Struma river, one from the Eastern Rhodopes and one from the Danube Plain); six populations of *Helichrysum arenarium* (two from the Black Sea coast and four from North-Eastern Bulgaria); three populations of *Primula veris* (Pirin Mt, in a pine forest above the village of Ilindentsi; Znepolski district, Golo Burdo above town of Pernik; Rhodope Mts, above Bachkovo) and three populations of *Arctostaphylus uva-ursi* (Vitosha, Kamen del locality, Pirin Mt, Betalovoto and Stara Planina, area "Kozyata Stena") were studied embryologically.

**Embryological study**

For the purpose of the embryological study, the collected material /flower buds and flowers at different developmental stages/ from populations of the target species was fixed in FAA and then embedded in paraffin. Subsequent processing was carried out according to the generally accepted classic paraffin method. Using a rotary microtome, paraffin sections with a thickness of 8 to 15 μm (depending on age and other specifics of the material) were made. Staining was performed with iron hematoxylin according to Heidenhain (1886). Permanent microscopic preparations were prepared by embedding the sections in Entelan. Observations were made with light microscope "Olympus" CX21. The photomicrographs were taken with an “Infinity lite” digital camera, 1.4 Mpx

**Assessment of pollen viability of the studied species**

The viability of the pollen was studied in connection with establishing the method of reproduction and the possibilities for realizing the reproductive capacity of the populations of the studied species. For this purpose, mature pollen grains were counted in 30 anthers for each of the studied populations of individual species.

Viability of mature pollen was examined by acetocarmine staining - acetocarmine test (Singh 2003) and scored according to the intensity of staining by the dye. The cytoplasm and nuclei of viable pollen grains are stained red, and non-viable and sterile ones are not stained.

**Assessment of seed (embryo) viability**

A tetrazole test was applied to assess embryo viability (Peters 2000). For this purpose, the seeds were incubated in a 1% solution of 2, 3, 5-triphenyltetrazolium chloride for 24 hours, according to a methodology developed by AOSA (Association of Official Seed Analysts). Initially, the solution of tetrazolium chloride is colorless, but subsequently changes its color from dark pink to red, as a result of the action of hydrogen ions released during the vital activity of the embryos (in the process of respiration). Embryos that are physiologically active are stained red and are counted as viable, and partially stained and non-stained embryos as non-viable. Before being treated with a 1% solution of tetrazolium chloride, the seeds were incubated for 24 hours in water at a temperature of 25 °C

**Laboratory seed germination testing**

Seed germination testing (stored at room temperature) was conducted in petri dishes on moistened filter paper in laboratory conditions

**Results of applied research methods**

**Embryological study**

On the basis of the embryological studies carried out, the embryological characteristics of the four studied species have been described: the features of the structures that make up the male /stamens/ and the female /pistil/ gametophyte have been described, as well as the way of the processes in them. The results showed that they were all sexually reproducing, through seeds, with the processes leading to the formation of pollen and seeds taking place correctly. Vegetative propagation is also present to varying degrees in individual species, most strongly in *A. uva-ursi*.

**Assessment of pollen viability**

Assessment of the viability of mature pollen using the acetocarmine pollen viability test showed that in all four species the majority of pollen grains were viable. The percentage of viability found was, respectively: between 95.27% and 98.67% in *A. tinctoria* populations; between 71.39% and 93.83% in *H. arenarium* populations; between 95.84% and 98.05% in *P. veris* populations and between 95.62% and 96.7% in *Arctostaphylos uva ursi* populations.

**Assessment of seed viability**

The seed viability assessement using the tetrazole viability test, demonstrated the following results:

- In *A. tinctoria* and *H. arenarium*, the viability of the seeds varies within relatively wide limits - respectively between 90% and 64% in the populations of *A. tinctoria* and between 4% and 61% in the populations of *H. arenarium*

- In the populations of *A. uva ursi* and *P. veris*, the variation is significantly narrower - between 43.75% and 53.3% for *A. uva ursi* and between 2% and 4% for *P. veris*, respectively

- Comparative testing of *P. veris* seeds collected from plants from natural populations of the species and from plants from the created *ex situ* collection showed a significant increase in the vitality of seeds collected from plants from the *ex situ* collection /up to over 60%/.

- The conducted comparative study of seed viability in different years showed that the amount of viable seeds changes in different years under the influence of climatic conditions /temperature, rainfall/, and this variation is greatest in the populations of *A. tinctoria* (up to 40 % in one of the populations)

**Seed germination**

Seed germination was tested under laboratory conditions, at room temperature, in petri dishes, on moistened filter paper. Germination under these conditions was reported only in the populations of *H. arenarium*, and in some of them it reached 80-96 %. However, most germinated seeds became moldy and died, and only about 8-9% of them developed into seedlings, which died when transferred from the filter paper to a soil substrate or coconut shavings. In *A. tinctoria*, only one seed germinated /from 30 planted seeds/ and only in some of the populations. The resulting seedlings were very small and extremely tender, and when transferred to a substrate for further growth, they died. The seeds of *P. veris* and *A. uva ursi* did not germinate at all, the former due to deep dormancy, the latter due to the hard seed coat.

**Conclusion:**

– For the first time, a study of basic characteristics of the reproductive biology (embryological features, pollen viability, seed viability and germination) of Bulgarian populations of the target species is being conducted, and for *Arctostaphylos uva ursi* and *Helichrysum arenarium* it is generally the first and as such is a contribution and enriches the embryological characteristics of the respective genus and family to which the studied species belong. The reproductive potential parameters /pollen viability, seed viability and germination/ are studied for the first time in all four species studied.

– The processes leading to the formation of pollen and seeds in the studied species proceed correctly, without significant deviations, which is a prerequisite for a high reproductive potential, which, together with the established high viability of the pollen, provides them with the opportunity to successfully renew the populations. The lower viability of seeds /compared to that of pollen/ is a result of the impact of environmental conditions on the quantity and quality of seeds produced.

- The established viability of the seeds is evidence of a satisfactory degree of realization of the reproductive potential, which depends on the environmental conditions and widely varies.
- The low germination of *Primula veris* and *Alkanna tinctoria* seeds and survival of *Helichrysum arenarium* seeds established under laboratory conditions is due to seed dormancy and physiological reasons
- The cultivation of *Primula veris* plants under controlled conditions /in *ex situ* collections/ significantly increases the vitability of the formed seeds /by about 60%/.

### *4.4. Work package 4. Genetic studies and flow cytometry*

WP 4 is represented by Assoc. prof. d-r Vladimir Vladimirov, PhD, a specialist in the field of flow cytometry, Prof. d-r Petar Zhelev, PhD, who is a specialist in the development and application of genetic markers to plant systems, Assoc prof. d-r Ina Aneva, PhD, a specialist in the field of conservation of medicinal plants and their genetic resourcese.

#### 4.4.1. Research methods and techniques

• Flow cytometry to determine the type of reproduction /sexual or apomyctic/ (Hojsgaard & Herbig 2013) and ploidy level control of colchicine-treated seeds and vegetative organs using a PARTEC CyFlow SLGreen liquid cytometer.;

• Genetic analyzes using DNA markers. A polymerase chain reaction (PCR) method is applied to amplify DNA fragments with two types of markers: microsatellites and ISSR

#### 4.4.2. Tasks according to the work program of the project

***1. DNA extraction and determination of DNA profiles of the studied species***

***2. Determination of genome size and ploidy level of embryo and endosperm by flow cytometry method***

#### 4.4.3. Results

**1. DNA extraction and determination of DNA profiles of the studied species**

Extraction was performed with Invisorb Spin Plant Mini Kit for P. veris and A. uva ursi, with Invisorb Spin Plant Mini Kit and modified CTAB protocol (Doyle & Doyle, 1990) for *A. tinctoria* and with modified cetyltrimethyl ammonium bromide polyvinylpyrrolidone (CTAB-PVP ) protocol in *H. arenarium* (Kramberger et al 2021). The quantity and quality of extracted DNA was checked using a spectrophotometer and by agarose gel electrophoresis (Barbas et al., 2007).

The dendrograms constructed on the basis of the genetic distances between populations, in all four studied species, showed that there is a correspondence between the geographical and genetic distances, which correspond to the distances between individual populations: in *P.veris* and *A.uva ursi*, the studied populations are clearly separated, since they fall into different geographical areas, in *A. tinctoria* the population from the Danube Plain is more clearly distinguished from the other populations from the Valley of Struma river, which are located closer to each other, while the populations of *H. arenarium* are not clearly distinguished from each other, as they are geographically very close.

The evaluation based on ISSR markers found, in all four studied species, close values of diversity indicators, which is an indication of a low level of genetic interpopulation variability.

The conducted genetic study of *in vitro* propagated plants of *H. arenarium* and hydroponically grown plants of *A. tinctoria*. showed that the genetic profile of such plants did not differ from that of the parent plants.

## 2. *Examination of the ploidy level of the embryo and endosperm by the method of flow cytometry*

The amount of nuclear DNA and the size of the genome (C-value) in the four studied species were determined using a liquid cytometer CyFlow SL Green of PARTEC, Germany. Treatment of the material (fresh leaves or seeds of the target species) was performed with CyStain PI Absolute P plant tissue kit manufactured by SYSMEX & PARTEC, following the protocol provided in the kit. C-values were determined by comparison to a standard, with an already known genome size value. To ensure equal treatment of the target plant and the standard, the two plant objects were mixed and processed together in the same tube (target + internal standard). Fresh leaves of *Pisum sativum* cultivar "Kleine Rheinländerin" (1C = 1Cx = 4.38 pg) or of *Solanum pseudocapsicum* (1C = 1.295 pg) were used as standards. The measured C-values of populations of individual species are very close, indicating stability of ploidy level within species populations, which in turn is an indication of stability in their reproductive type.

The analysis of the type of reproduction in different growing seasons showed comparable C-values in different growing seasons in all four species, viz. preservation of the reproductive type.

During the cytometric study of plants from the studied populations of *H. arenarium* obtained by *in vitro* cultivation, no deviations in the ploidy level were detected compared to that of the natural populations. This indicates that in vitro propagation is a suitable method for cultivating *H. arenarium* , since the size of the genome does not change, the ploidy level of the starting plants, respectively their quality, is preserved.

**Conclusion:**

- A low level of genetic variability was found between the populations of the studied species, which presupposes limitations to their distribution and greatly contributes to the fragmentation of their populations and limitation in the range of distribution
- No variation in ploidy level was found between populations of the target species.
- *In vitro* propagated plants from *H.arenarium* and hydroponically grown from *A. tinctoria* retain the genetic profile and ploidy level of the parent plants.
- The observed low genetic variability and comparable C-values during individual growing seasons in all four species are an indication of stability of their type of reproduction.
- The method used for *in vitro* propagation of *H. arenarium* by direct organogenesis is a suitable method for cultivating not only this species, but also other valuable wild plant species, since its application does not change the genome size, preserves the ploidy level of the starting plants, respectively their quality.

***4.5. Work package 5 Plant biotechnology and hydroponics:***

WP 5 consists of young scientists and doctoral students led by Prof. d-r Marina Stanilova, who founded the Biotechnological Laboratory for Medicinal Plants, within the the international project NATO SfP 974453-Bioproduction (2001-2006) led by her and developed the field of plant biotechnology at IBEI. Prof. Stanilova has experience in the management of graduate students, doctoral students and number of projects, incl. funded by FNI related to *in vitro* propagation of medicinal, rare and endemic plants. Assist. prof. Maria Petkova has experience in the *in vitro* cultivation of valuable medicinal plants such as *Arnica montana*. Doctoral students Asya Kojuharova and Vladimir Ilinkin have very good results in clonal reproduction, and the young scientist Boryanka Traikova - in *ex vitro* adaptation, maintenance of *ex situ* collections and hydroponic cultivation.

**4.5.1. Research methods and techniques**

• Initiation of *in vitro* culture from seeds (George 1996).
• Clonal micropropagation (George 1996).
• Somatic embryogenesis (George 1996).
• Rhizogenesis and hairy root cultures (Giri & Narasu 2000).
• Increasing plant ploidy level by colchicine treatment of seeds and vegetative organs *in vitro* (Mori et al.; 2016; Henny & al. 2009). Tetraploid plants are larger in size, and in some medicinal plants a significant increase in the content of BAS synthesized by them has been noted.
• Ex vitro adaptation and acclimatization of *in vitro* rooted plants (George 1996) by planting in pots with a soil substrate, their adaptation to normal conditions being carried out step by step: in a climate chamber (POL-EKO Aparatura) with strict control of humidity, temperature and lighting, simulating their daily fluctuations, with a gradual decrease in humidity from 90 to 60% for 4 to 6 weeks, then in a phytotron room with controlled conditions over a wider range. Acclimatization is carried out in a greenhouse, after which the plants are planted in experimental areas.
• Hydroponic /soilless/ cultivation (Sardare & Admane 2013).: from seeds or from vegetative organs, using available hydroponic (Cutting Board) and aeroponic (Green Diamond, GH) systems, as well as a new hydroponic system (Aero Flo-14 ). The resulting plants are acclimatized in a greenhouse and planted in test areas

**4.5.2. Tasks according to the work program of the project**

***1. Initiation of in vitro cultures from seeds or from vegetative plant organs;***
***2. Stimulation of seed germination in vivo and in vitro***
***3. Obtaining in vitro plants by direct and indirect regeneration and determining the influence of growth regulators, sub-cultivation techniques and environmental parameters (temperature, lighting)***
***4. Adaptation of in vitro obtained plants to environmental conditions***
***5. Cultivation of target species in hydroponic systems.***

### 4.5.3. Results

#### 4.5.3.1. Initiation of *in vitro* cultures from seeds or from vegetative plant organs

Attempts were made to obtain seedlings *in vitro* by seeds germination on basic MS medium. Only in *Helichrysum arenarium* was a relatively high seed germination was reported, and in this species, despite significant microbial contamination, *in vitro* cultures of 5 populations of the species were successfully initiated. In the seeds of the other species, germination was problematic: only one seed germinated from several hundred *in vitro* set seeds of *Primula veris*, and in *Alkanna tinctoria* and *Arctostaphylos uva-ursi* no germinated seeds were noted.

Various methods were applied to stimulate seeds germination, such as: pre-treatment with low temperature, gibberellic acid for different periods of time, or placing on MS medium containing gibberellic acid, but none of these methods gave results. In addition, an attempt was made to initiate an *in vitro* culture of embryos in *A. tinctoria*, but it was also unsuccessful.

Difficulties in seed germination of *A. tinctoria* and *Primula veris* were overcome in vivo thanks to a wide variation scheme combining seed pretreatment with gibberellic acid (GA3) and 12 h/day monochromatic light irradiation (Fig. 14, 15).

*Figure 14 Germination of Alkanna tinctoria seeds under in vivo conditions in petri dishes under monochromatic light application - from left to right: white, infrared, red, blue.*

*Figure 15 Germination of Primula veris seeds under in vivo conditions in petri dishes,*

### 4.5.3.2. Obtention of *in vitro* plants

*In vitro* plants were obtained only in *Helichrysum arenarium* by direct organogenesis on MS basal medium without growth regulators and with 20 g/l sucrose, from in vitro germinated seeds from natural populations of the species (Figs. 16.1 and 16.2). When BAP and NAA were added to the medium, callus formation was also observed, which on sub-cultivation grew rapidly and plants formed, but in most cases vitrified (Figs. 16.3 and 16.4).

*Figure 16 1) and 2) Formation of normally grown plants by direct organogenesis on 3/4MS-20 medium, 3) multiplication accompanied by strong vitrification in most explants 4) indirect organogenesis on callus subculturing*

Seedlings obtained from the *in vitro* germinated seeds of *Primula veris*, collected from plants growing in the *ex situ* collection, formed only rudiments of roots and were not suitable for *ex vitro* adaptation /Fig. 17/

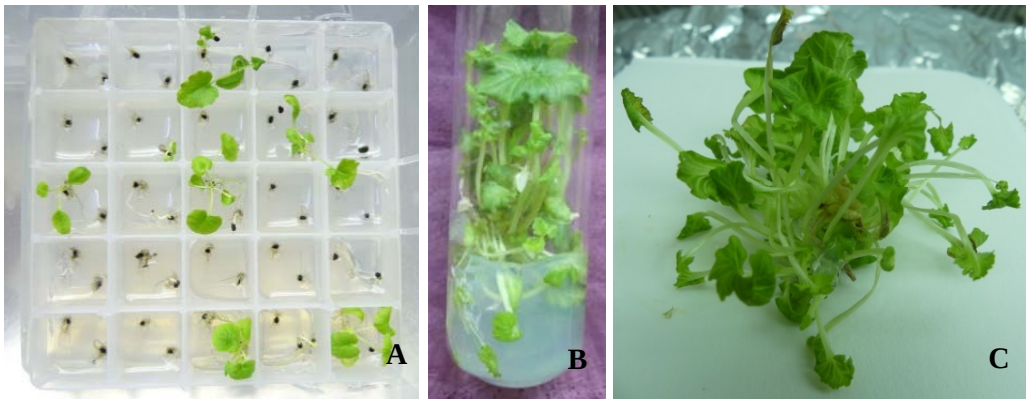


*Figure 17. In vitro cultivation of P. veris: A – seed germination on medium containing gibberellic acid and kinetin; B, C – young plants obtained after subcultivation.*

#### 4.5.3.3. Adaptation of *in vitro* obtained plants to environmental conditions

The obtained (*in vivo*, *in vitro* and hydroponically) of *Alkanna tinctoria*, *Helichrysum arenarium* and *Primula veris* were successively transferred to a substrate of soil and perlite/vermiculite in a phytotron room, a greenhouse and an experimental field for adaptation to environmental conditions. The plants of all three species adapted successfully /Fig.18, 1-3/.


*Figure 18.1. Acclimatization of hydroponically grown A. tinctoria plants: A) Well-developed rosettes transferred to pots with soil substrate in the phytotron room; B) Plants with branched stems in the greenhouse; C) Plants in the ex situ collection of IBEI; D) Beginning of flowering at the end of May 2020*


*Figure 18.2. Ex vitro adapted plants of H. arenarium: A) in a climate cabinet; B) in a phytotron room*

*Figure 18.3. Adaptation and cultivation of Primula veris plants in phytotron (on vermiculite and in soil mixture)*

### 4.5.3.4. Cultivation of target species in hydroponic systems

In the present project, through the techniques of hydroponic cultivation, plants obtained from pre-stimulated seeds of *A. tinctoria* /on the Cutting board hydroponic system were successfully grown - Fig. 19/

The testing of the possibilities of reproduction by hydroponic technologies was also carried out on cranberry (Vaccinium vitis-idaea L.), used as a reference species, from the same family, also included in the List of species of medicinal plants under a special regime of protection and use in Bulgaria, based on the Medicinal Plants Act (2000), participating with Bearberry in threatened habitat type 09F2 Highland Communities. Like bear grapes, it shows slow growth and development. Unlike bear grapes, where hydroponic cultivation did not prove to be a suitable technique, in cranberry, the Flood & Drain (F&D) type hydroponic system used rooted 32 cuttings, 62.5% acclimatized under greenhouse conditions, grew successfully and reached yes flowering phase /Fig.20/.


*Figure 19. Plants of A. tinctoria with a powerful root system, placed on the Cutting board hydroponic system: A) top view; B) bottom view; C) whole plant ready to transfer from the hydroponic system to a pot with soil substrate*

*Figure 20. Bearberry cuttings on a Flood & Drain hydroponic system*

**Conclusion:**

- A successful technology has been developed to grow *A. tinctoria* in culture by combining pre-stimulation of seed germination and hydroponic cultivation of the resulting seedlings. The established pilot culture in the experimental areas of IBEI can serve as a basis for the development of effective protocols for the cultivation of the species under controlled conditions.

- The extremely low germination rate of *P.veris* seeds was overcome by prestimulating the seeds by treating them with a combination of stimulating factors. Based on this, a protocol for cultivating the species was established.

- A protocol for the in vitro cultivation of *H. arenarium* has been established. The method of regeneration through direct organogenesis turned out to be optimal. This method is suitable for application for the rapid propagation of the species for the purposes of growing it in culture, since the resulting plants retain their original ploidy level.

- The conditions for the application of hydroponic growing techniques for shrubby plants have been established.

## 5. Summary

During the implementation of the activities of this project, the following results were obtained:

- The distribution of *Alkanna tinctoria* and *Helichrysum arenarium* in Bulgaria has been established.
-Field studies proved the need to cultivate the target species in order to provide raw material for the pharmaceutical industry.
-*Ex situ* collections of the studied species have been created in the greenhouse and experimental areas of IBEI BAS and AU Plovdiv, which can serve as a source of starting material for their cultivation.
-The metabolic profile of the populations of *Alkanna tinctoria* from different phytogeographic regions of Bulgaria: Valley of Struma river, Danube Plain and Eastern Rhodopes was determined. The amount of alkannin in the individual populations was identified and compared with that of the commercial product-roots of *A. tinctoria*, which in the populations of the Valley of Struma river is close to and even exceeds the amount found in the commercial product.
-A comparative study of the chemical profile of the aerial parts of *A.tinctoria* was conducted, which is the first of its kind.
-The presence of ononitol in the aerial parts of the species, as well as phenolic acid, in the aerial parts and in the roots of *A.tinctoria* is reported for the first time
- The qualitative and quantitative composition of the essential oil of the Bulgarian populations of *Helichrysum arenarium* was established. Their chemical profile was assessed as a new chemotype for the species, with main components α-pinene (34.64–44.35%) and sabinene (10.63–11.1%).
-A comparative analysis of the essential oil composition of *H. arenarium* with that of *H. italicum* was made, which showed that both species have a specific essential oil profile: monoterpenes (α-pinene, sabinene) are the predominant class of compounds in the essential oil of *H. arenarium*, while the essential oil of *H. italicum* is dominated by sesquiterpenes (neryl acetate and β-hymahalene).
-The exudate flavonoids from the leaves of *Primula veris* were identified and their capacity as a source of biocidal activity /as inhibitors of weed seed germination on crop plants/ was evaluated.
-Inhibitory activity of flavonoids isolated from flowers of *Helichrysum arenarium* on the germination of *Lolium perenne* L. /a weed on cultivated plants/ has been established, which supports similar results with exudates from other aromatic and essential oil plants and reinforces the conclusion that exudates from such plants can also be successfully applied as agents with biocidal activity in the control of weeds on crop plants.
- The bioactive compounds in the methanolic extracts of leaf samples from Bulgarian populations of *A. uva ursi* were identified.
-The genetic profile of selected populations of the target species has been established, which does not show high inter-population variability in all four studied species.
- The size of the genome in the four target species was determined, which does not change in the individual populations and in the four studied species and is an indication of the stability of their ploidy level.

-The reported genome size for *H. arenarium* is the first report for the genome size not only for Bulgarian populations, but for the species in general.

- The features of the structures and processes leading to the formation of pollen and seeds, which are a prerequisite for the successful reproduction of the populations of the studied species and the respective preservation of their sizes, have been revealed.
- An assessment was made of the reproductive potential of the populations of the target species, which is affected by the change in environmental conditions. .
- The obtained results of the genetic profile and established features of reproductive biology show that the limited distribution of *A. tinctoria* and *H. arenarium* in Bulgaria is the result of the low genetic variability of the populations of both species and low plasticity of the reproductive apparatus, which determine a low adaptability to environmental conditions.
- A protocol for *in vitro* propagation of *H.arenarium* by direct organogenesis has been established. It is a suitable method for the cultivation not only of this species, but also of other valuable wild-growing plant species, since its application does not change the size of the genome, the ploidy level of the starting plants, respectively their quality, is preserved.
- The conditions for *ex situ* cultivation of the four target species have been established as follows:
- *Alkanna tinctoria* – by hydroponic cultivation
- *Helichryzum arenarium* – by introduction into culture *in vitro*
- *Arctostaphylus uva-ursi* – by cuttings
- *Primula veris* – by stimulating seed germination *in vivo*

For three of them, protocols for cultivation in agriculture have been developed:

- *Alkanna tinctoria*: propagation by seeds treated with gibberellic acid and monochromatic light to stimulate germination; transferring the obtained seeds onto a Cutting board hydroponic system for further development in young plants; adaptation of young plants to a soil substrate in pots, successively in a phytotron room and in a greenhouse (to strengthen and form branched stems); transferring and planting the plants outdoors.
- *Helichryzum arenarium*: propagation in *vitro* from seeds on MS basic nutrient medium free of growth regulators and with 30 g/l sucrose; transferring the obtained seedlings to medium MS-20 (without growth regulators) and with 20g/l sucrose, for further growth and root formation; adaptation of young plants to a soil substrate in pots, successively in a phytotron room and in a greenhouse; transferring and planting the plants outdoors.
- *Primula veris*: stimulation of seed germination *in vivo* by their combined treatment with $GA_3$ and monochromatic light; adaptation of the obtained young plants to a soil substrate in pots, successively in a phytotron room and in a greenhouse; transferring and planting the plants outdoors.

**The results of the conducted research are presented in:**

- **11 papers**:
- – **10 printed**:

Boryanka D. Traykova, Irena D. Grigorova, Marina I. Stanilova, Emil D. Molle**,** Elina P. Yankova-Tsvetkova. 2020. *Alkanna tinctoria*: An Approach Toward Ex situ Cultivation. Ecol. Balk. Special Edition 3: 107-115. SJR 0.14 **Q4**
http://web.uni-plovdiv.bg/mollov/EB/2020_SE3/107-115_eb.20SE303.pdf
https://doi.org/10.3390/plants12010111

Elina Yankova-Tsvetkova, Stoyan Stoyanov, Antonina Vitkova, Ivanka Semerdjieva. On the Distribution of *Helichrysum arenarium* (Asteraceae) in Bulgaria. 2021. Compt. Rendus Acad. Sci. Bulg 74(11): 1599-1606 . SJR (Scopus):0.19 **Q3** IF: 0.329
https://www.proceedings.bas.bg/index_old.html

Yankova-Tsvetkova, E., Yurukova-Grancharova, P., Aneva, I., Zhelev, P. 2021. On the reproductive potential in *Primula veris* L. (Primulaceae): Embryological Features, Pollen and Seed Viability, Genetic Diversity. Plants 10(11) 2296 ISSN: 2223-7747, **Q1**, IF 4.67 (2021)
https://doi.org/10.3390/plants10112296

Elina Yankova-Tsvetkova, Petka Yurukova-Grancharova, Rozalia Nikolova. 2022. On the Reproductive Biology of *Alkanna tinctoria* (Boraginaceae) Compt. Rendus Acad. Sci. Bulg 75(7): 1000-1008 SJR (Scopus):0.19 **Q3** IF: 0.329
https://www.proceedings.bas.bg/index.php/cr/issue/view/7

Zheljazkov, V.D.; Semerdjieva, I.; Yankova-Tsvetkova, E**.;** Astatkie, T.; Stanev, S.; Dincheva, I.; Kačániová, M. Chemical Profile and Antimicrobial Activity of the Essential Oils of *Helichrysum arenarium* (L.) Moench. and *Helichrysum italicum* (Roth.) G. Don. Plants 2022, 11, 951. IF 4.658 **Q1**
https://doi.org/10.3390/plants11070951

Marina Stanilova, Boryanka Traykova, Vladimir Vladimirov, Maria Petrova, Ivanka Semerdjieva, Elina Yankova-Tsvetkova. 2022. In vitro Micropropagation of *Helichrysum arenarium* (Asteraceae) as a Tool for Introducing the Species in Agriculture. Compt. Rendus Acad. Sci. Bulg 75(10): 1454-1461 SJR (Scopus):0.19 **Q3** IF: 0.329
https://www.proceedings.bas.bg/index.php/cr/issue/view/10

Yankova-Tsvetkova, E**.;** Petrova, M.; Grigorova, I.; Traykova, B.; Stanilova, M. The Establishment of an Ex Situ Collection of *Primula veris* in Bulgaria. Plants 2022, 11, 3018. IF 4.658 **Q1**
https://doi.org/10.3390/plants11223018

Nikolova, M.; Aneva, I.; Zhelev, P.; Semerdjieva, I.; Zheljazkov, V.D.; Vladimirov, V.; Stoyanov, S.; Berkov, S.; Yankova-Tsvetkova, E. Metabolic Profiles, Genetic Diversity, and Genome Size of Bulgarian Population of Alkanna tinctoria. Plants 2023, 12, 111. https://doi.org/10.3390/plants12010111

Nikolova M, Yankova-Tsvetkova E., Stefanova, T., Stoyanov, S., Berkov S. Evaluation of Helichrysum arenarium flowers exudate as inhibitor on Lolium perrene seed germination under laboratory conditions.2023. Acta Agrobotanica, Acta Agro 2023;76,
DOI: https://doi.org/10.5586/aa.761

Milena Nikolova, Elina Yankova-Tsvetkova, Tatyana Stefanova, Strahil Berkov 2023. Exudate flavonoids of Primula veris leaves and their inhibitory activity on Loliun perrene seed germination. Comptes rendus de l'Acad´emie bulgare des Sciences, 76, 3, 388-393 https://www.proceedings.bas.bg/index.php/cr/article/view/272/264

**1 in press**

Milena Nikolova, Ina Aneva, Elina Yankova-Tsvetkova, Petar Zhelev, Strahil Berkov 2023. Bioactive compounds of Arctostaphylos uva-ursi wild growing populations from Bulgaria. Biological Communication

- **2 short communications**:

Elina Yankova Tsvetkova, Stoyan Stoyanov, Antonina Vitkova, Kiril Vassilev, Ivanka Semerdjieva. Notes on the distribution of Alkanna tinctoria (L.) Tausch in Bulgaria Macedonian Pharmaceutical Bulletin, 66 (Suppl 2) 23 - 24 (2020) Online ISSN 1857 – 8969 UDC: 582.929.2-19(497.2)
DOI: 10.33320/maced.pharm.bull.2020.66.04.011 Short communication

Marina Stanilova*Boryanka Traykova, Irena Mincheva, Elina Yankova-Tsvetkova. Soilless propagation of wild Ericaceae medicinal shrubs. Macedonian pharmaceutical bulletin, 68 (Suppl 2) 213 -214 (2022) Online ISSN 1857 – 8969
DOI: 10.33320/maced.pharm.bull.2022.68.04.098 Short communication

- **1 book**

Yankova-Tsvetkova, E. (ed). 2022. Cultivation of medicinal plants species from the Bulgarian flora with resource deficit. Intel Entrance Ltd, Sofia, p. 48 Sofia 2022, ISBN:978-619-7703-07-8.

- **13 posters in 10 scientific forums:**

**1.**13th Symposium on the Flora of Southeastern Serbia and Neighboring Regions, Stara Planina Mt., 20-23 june 2019- **1 poster**

1.Yankova-Tsvetkova, E., Berkov, S., Stanilova, M., YurukovaGrancharova, P., Vitkova, A., Nikolova M., Zhelev, P., Stoyanov, S., Vladimirov, V., Aneva, I., Kozhurahova, A. ,Traykova, B., Ilinkin, V.1, Nikolova, R., Stefanova, T., Dimitrova, M.1, Semerdjieva, I., Zheljazkov, V. Reproductive potential, metabolic and genetic profile, in in situ and ex situ conditions, of medicinal plants species from the bulgarian flora with resource deficit – scientific base for their cultivation. In Book of Abstracts 13th Symposium on the Flora of Southeastern Serbia and Neighboring Regions, Stara planina Mt., 2019, P.123-124.

**2.**5th International Congress On Biodiversity: Taxonomy, Speciation And Euro-Mediterranean Biodiversity, 11-13 october 2019 Sofia - **1 poster**

2.Elina Yankova-Tsvetkova, Stoyan Stoyanov, Antonina Vitkova, Ivanka Semerdjieva. On the distribution of Helichrysum arenarium (L.) Moench (Asteraceae) in Bulgaria
https://aca.pensoft.net/article/46489/

**3**.8th International Conference of Ecologists of Montenegro, 2-6 october 2019, Budva, Montenegro – **1 poster**

3.Milena Nikolova, Elina Yankova-Tsvetkova, Vladimir Ilinkin, Strahil Berkov. GC/MS metabolite analysis of the aerial parts of *Alkanna tinctoria* Book of Abstracts ISEM8 October, 2-5, 2019 p.91

**4**. International Seminar of Ecology 2020 On line seminar 23-24 april 2020 г. – **1 poster**

4. Traykova, B., Grigorova, I., Stanilova, M., Molle, E., Yankova-Tsvetkova, E. 2020. *Alkanna tinctoria*: an approach toward ex situ cultivation. Book of Abstracts. International Seminar of Ecology 2020, p. 32

**5**. II International Agricultural, Biological & Life Science Conference (AGBIOL) September 1-3 2020, Edirne, Turkey – **1 poster**

5. Nikolova, M., Yankova-Tsvetkova, E., Vitkova, A., Stojan, S., Semerdjieva, I., Berkov, S. Assessment of alkannin content in the roots of Bulgarian populations of Alkanna tinctoria. Book of Abstracts. II International Agricultural, Biological & Life Science Conference (AGBIOL) September 1-3, 2020, Edirne, Turkey p. 351л

**6**.5th Balkan Scientific Conference on Biology, Plovdiv, 15-16 april 2021 - **2 posters**

6. Yankova-Tsvetkova, E., Yurukova-Grancharova, P, Nikolova, R. 2021. A study on reproductive capacity in Primula veris L. (Primulaceae). https://zenodo.org/record/4641763#.Y_S0NXZByUk

7. Stanilova, M., Petrova, M., Grigorova, I., Traykova, B., Yankova-Tsvetkova, E**.** 2021. Stimulation of *Primula veris* seeds'germination under in vivo and in vitro conditions https://zenodo.org/record/4650322#.Y_S1d3ZByUk

**7**.1st International Conference on Botany and Mycology, Sofia 25–26 October 2021 - **1 poster**

8. Stanilova, M., Traykova, B., Vladimirov, V., Petrova, M., Yankova-Tsvetkova, E**.** 2021 *In vitro* micropropagation of Helichrysum arenarium (Asteraceae) as a tool for introducing the species in agroculture. Book of Abstracts. 1st International Conference on Botany and Mycology, Sofia 25–26 October 2021, p. 43 https://icbotmyco.com/wp-content/uploads/2021/10/BOTMYCO-2021-Abstracts-Book.pdf

**8.**8th Balkan Botanical Congress, 4-8 July 2022, Athens, Greece: - **2 posters**

9. Nikolova M.*, Aneva I., Yankova-Tsvetkova E**.,** Berkov S. Bioactive compounds of *Arctostaphylos uva-ursi* growing in Bulgaria. 8th Balkan Botanical Congress, 4-8 July 2022. Book of abstracts, pp: 76.
10. Semerdjieva I.1,2*, Zheljazkov V.D.3, **Yankova-Tsvetkova E**., Dincheva I., Astatkie T. Chemical profile of essential oil of Helichrysum arenarium (L) Moench. distributed in Bulgaria. 8th Balkan Botanical Congress, 4-8 July 2022. Book of abstracts, pp: 79. https://www.uoa.gr/fileadmin/user_upload/PDF-files/ekdilwseis/Synedria/2022/0107_balkan_botanical_congress_programma.pdf

**9.**2nd International Conference on Botany and Mycology, 19–20 September 2022, Sofia, Bulgaria - **- 2 posters**

11. Elina Yankova-Tsvetkova* & Rozalia Nikolova. On the reproductive biology of *Arctostaphylus uva-ursi* (Ericaceae). 2nd International Conference on Botany and Mycology, 19–20 September 2022. Book of abstracts, p. 23.
12. Milena Nikolova, Elina Yankova-Tsvetkova***,** Tatyana Stefanova, Marina Dimitrova, Strahil Berkov. Exudate compounds of *Primula veris* leaves and their biological activity 2nd International Conference on Botany and Mycology, 19–20 September 2022. Book of abstracts, p. 34.
https://icbotmyco.com/wp-content/uploads/2022/09/BOTMYCO-2022-Abstracts-Book.pdf

**10**.11th Conference on Medicinal and Aromatic Plants of Southeast European Countries 2022 (CMAPSEEC 2022), 6-10 October 2022 in Ohrid, Republic of North Macedonia – **1 poster**

13. Marina Stanilova*, Boryanka Traykova, Irena Mincheva, Elina Yankova-Tsvetkova. Soilless propagation of wild Ericaceae medicinal shrubs, 11th Conference on Medicinal and Aromatic Plants of Southeast European Countries 2022 (CMAPSEEC 2022), 6-10 October 2022 in Ohrid, Republic of North Macedonia, Book of abstracts, pp: 213-214.
http://cmapseec.mfd.org.mk/wp-content/uploads/2022/12/Supplement-Issue_68_2_2022_CMAPSEEC.pdf

**During the field research in connection with the implementation of the tasks of WP1, a species new to the Bulgarian flora was found, which is described in the following publication, with thanks to the project::**

Stoyan Stoyanov, S., Barzov, Zh. 2020. *Papaver nigrotinctum*: first records for the flora of Bulgaria and Republic of North Macedonia. – Phytologia Balcanica 26(1): 35–42.