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Conservation of *Leucojum aestivum* (*Amaryllidaceae*) in Bulgaria

Abstract

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The nature protection status and measures of conservation of *Leucojum aestivum* L., an economically important species as a pharmaceutical crude drug, are presented. The main threats of the natural populations in Bulgaria were identified. Background information concerning chorology, ecology and resources of the species were summarized. *In situ* conservation approaches were discussed: including the population *status* in protected areas, recommendations for their management and sustainable use of the resources. The experience in *ex situ* conservation of *L. aestivum* was summarized. Application of biotechnological methods as well as *in vivo* rapid propagation via twin-scaling method were assessed and envisaged to be developed as an alternative of natural population use. Current status of 17 galanthamine-type populations localized in Southeast Bulgaria was described. Bulbs and seeds were gathered as a germplasm for *ex situ* conservation.

Introduction

Summer snowflake (*Leucojum aestivum* L.) is an economically valuable species and one of the most promising sources for production of galanthamine (Gal), an Amaryllidaceae alkaloid with wide-scale therapeutic application. Its anticholinesterase activity is employed for treatment of several nervous disorders and its analeptic effect can be clinically used for anaesthetic purposes. Bulgarian pharmacologists and phytochemists were among the first to create and produce Gal preparations (Paskov 1955; Ivanova & Ivanov 1959), when initially *Galanthus nivalis* L. was used as a Gal source. The advent of *Leucojum aestivum* (Ivanova & Ivanov 1959) as the most productive Gal source initiated the utilization of this species. The good therapeutic properties of Bulgarian preparations (Nivalin®, Nivalet®, Nivalin P®, Nivatonin®) expanded their market and increased the demand of crude drug. About 10 to 15 t of herbage were obtained annually from the natural *Leucojum* populations in Bulgaria during the 1960-1970 period, with a subsequent decrease of the yields to 5 t (Mitrev 1995). Then the natural resources were extremely limited and failed to meet the needs of pharmaceutical industry. Gal has been patented for treatment of alcohol and nicotine dependence (Moormann 1999) as well as Alzheimer's disease (Davis 1987). This intensified exploration for Gal sources. The expensive and unprofitable artificial Gal synthesis is no alternative for the natural sources (Czollner & al. 1998). Thus the question

of conservation of *Leucojum aestivum* resources came urgently to the fore, as well as of the perspectives of their use.

Human activities entailing destruction of habitats and excessive exploitation of the resources put *Leucojum aestivum* in the Red Data Book of Bulgaria (Velchev 1984) in the category "Endangered species". It was subjected to detailed studies aiming at solving the resource and conservation problems of this valuable species.

Chorology, habitats and ecology of *Leucojum aestivum* in Bulgaria

The Euro-Mediterranean species *Leucoium aestivum* occurs in low places along some of the major rivers in Bulgaria and their tributaries: Danube, Maritza, Tundzha, Kamchia, Ropotamo, Dyavolska, etc. According to floristic regionalization, the main localities belong to the following floral regions: the Forebalkan, Danubian plain, Thracian Lowland, Tundzha Hilly Country, the Black Sea Coast, Mt Vitosha, Northeastern Bulgaria (Kozhuharov 1992).

Leucoium aestivum is a hygromesophyte, bulbous ephemeroide, inhabiting inundated or marshy meadows, swampy terrains, thinned riparian forests of longos type, at an altitude of 0-200 (300) m. As an element of the potential vegetation it occurs as an autochthonous assectator, seldom as an edificator in hygro- and hygromesophylic communities. It is typical for the riparian tree communities *Alneta glutinosae* and *Fraxineta oxycarpae* relating to the conservationally valuable habitat of Coastal Bulgarian Longos Forest and to the inundated grass communities of *Carex ssp. diversa, Typha ssp.*, *Phragmites australis*, etc.

The vegetation period of *L. aestivum* begins in October-November, followed by compulsory dormancy and wintering. It resumes its growth in February-March and the active plant development is in April, when in 30 days it undergoes three phenophases: budding, flowering and fruit-bearing. In July herbage dries and bulbs fall into physiological summer dormancy. Mesoecological and edaphic peculiarities determine the fluctuation of the flowering stage about 25 to 30 or more days. According to Stefanov (1990), populations of *L. aestivum* are relatively old (50-60 years) and the vegetative propagation (bulblets formation) is more frequent than seed propagation.

Summer snowflake and anthropogenic influence: problems and threats

The analysis of literature data (Stoyanov & Savchev 1964; Astadjov 1969; Stefanov 1990; Mitrev 1995) and available information in the Minstry of Environment and Waters concerning populations monitored during last decade, outlined that following human activities present problems and threats for the populations and habitats of *L. aestivum*: draining of inundated and marshy terrains, incorrect afforestation practices, urbanization and assimilation of terrains for development of tourism infrastructure, incorrect gathering and overutilization of resources, haymaking and grazing. Monitoring of some populations in 2001 (Table 1) determined the negative effect caused by anthropogenic factors: *on habitat level:* destruction of habitats and irreversibly changed ecological conditions, drainage of inundated forests (longos) (along river Tundzha), replacement of natural forest vegetation with introduced one, draining of wet meadows and turning them into arable land; *on*

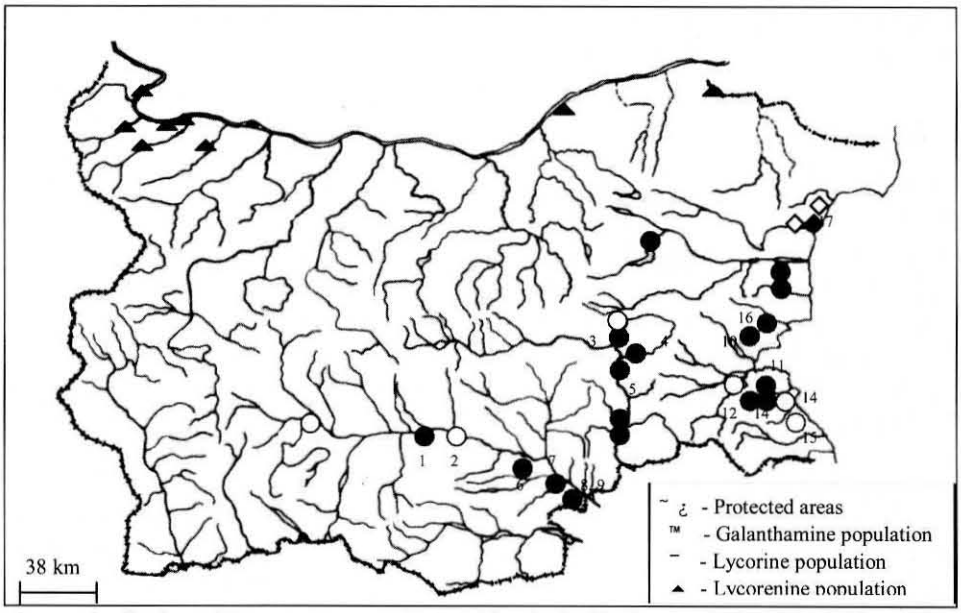

Fig. 1. Distribution of *Leucojim aestivum* L. in Bulgaria (in black - population included in protected area; for numbers see Table 1.)

population level: extinction of local populations in different regions of the country, shrinking down of areas occupied by *Leucojum* populations, disturbance of spatial and age structure of the populations, changes in the life cycle, decrease of productivity of populations and loss of their economic importance.

Resource characteristic

Use of *L. aestivum* for industrial purposes requests qualitative and quantitative assessment of its resources.

The first inventory of *L. aestivum* populations was made by Stoyanov & Savchev (1964). They described 48 localities on 1585 ha of general plot area. The productivity of dry herbage of the 30 Gal populations (1433 ha) was 28 kg/ha. Five years later Astadjov (1969) reported considerable shrinking down of the areas ocuppied by *L. aestivum* populations and extiction of the species in several localities. The total area of observed localities was 136 ha or 8 times lower than in 1964. The next assessment of resources (Stefanov 1990) showed only 27 localities in the country and 20 of them were of the Gal type. The potential biomass production reached 5 t, i.e. 3 times lower than in the 1960-1975 period.

L. aestivum populations differ in alkaloid quantity and composition and, according to the main alkaloid, three chemoraces are distinguished: Galanthamine, represented by 20 populations located in Southeast Bulgaria; Lycorenine: six populations along river Danube; Lycorine: one population situated in Baltata, North Black Sea Coast (Fig. 1).

Source of pharmaceutical crude drug are Gal-type populations in Southeast Bulgaria

along rivers Maritza and Tundzha, the Black Sea Coast and Kamchia. The Gal content varies from 0,10% to 0,56% (Stefanov 1990).

In situ conservation

The first *in situ* conservation measure applied to *L. aestivum* was protection of its habitats. Large areas of ecosystem complexes of longos type were proclaimed nature reserves: Kamchia, Baltata, Arkoutino, Gorna and Dolna Topchia. In 1970 the Ministry of Forests and Forestry gave the statute of protected areas to 11 localities of *L. aestivum,* where tillage was forbidden, while grazing and herb-gathering were restricted according to a species-specific phenology. The protected localities were fenced off, but the main factors affecting the environmental conditions of *L. aestivum* were not eliminated: draining of adjoining terrains and changes of hydrological regime. The protection was formal and difficulties did not find a practical solution. The Gorna and Dolna Topchia Reserves near river Tundzha were irreversibly affected. The status of many protected localities, such as Vinitza, Palauzovo, Debelata Koria, Bisser, Lozenski Pat, Ormana, had worsened. In order to implement their function of conservation of species as a genetic resource and economic reserve, the protected areas required effective management and practical decision-making.

To improve management of *Leucojum* resources, a restrictive regime of use according to the status of populations was prescribed to the species in 1981. The Ministry of Environment and Waters launched annual estimation of resource capacity for herb gathering and approved quotas for each population. However, it was difficult to regulate the norms of gathering so as to guarantee sustainability of resources. Educational and qualificational activities to enhance nature-conservation awareness of herb gatherers and buyers were not carried out. Possibilities for effective control during gathering campaigns was limited owing to lack of funds.

Usage of resources in the last six years was minimized and since 1998 herbal drug has not been harvested. Populations could be used only as a genetic material source. The restricted regime and monitoring system are still in place, accompanied by positive legislation changes: the Law on Medicinal Plants (2000) and the Law on Protected Areas (1999). Management plans for some important reserves, including *Leucojum* localities, are now in the process of development: in Kamchiya, Gorna and Dolna Topchia, and Arkoutino. Adequate programs and projects are envisaged within the framework of these schemes. Urgent conservation and reclamation measures, as well as strict control of the management plans for the remaining localities of *L. aestivum* are needed: restoration of the ecohydrological regime in habitats, optimization of population density by replanting and resowing with seeds and bulbs of the same origin, multiplied under conditions ensuring their authenticity.

Ex situ conservation

Difficulties to ensure sustainable use and effective conservation of natural *Leucojum* populations require measures for *ex situ* conservation and alternative approaches to obtain Gal source: *in vivo* and *in vitro* cultivation. Intensive research activities were launched, starting with studies on introduction and agrobiology of the species. Investigations on

Table 1. Current status of seventeen *L. aestivum* populations localized in Southeast Bulgaria.

No	Locality	Administrative region	Category of the protected area	Habitat	General plot area (ha)	Density (ind/0.25m^2)			Vitality and Fertility	Human impact
						generative	vegetative	general		
1	Vinitza	Plovdiv	PL	fluvial forest	9	-	-	-	3	B; C; E
2	Gradina	Plovdiv	unprotected	fluvial forest	20	2,02 ± 0,60	7,40 ± 1,18	9,42 ± 1,40	2	A; C
3	Ormana	Stara Zagora	PL	fluvial forest	30	1,45 ± 0,24	3,85 ± 0,51	5,29 ± 0,64	1	E
4	Palauzovo	Stara Zagora	PL	wet meadow	16	0,97 ± 0,19	0,88 ± 0,22	1,84 ± 0,36	2	B; E; A
5	Debelata koria	Stara Zagora	PL	wet meadow	15	0,79 ± 0,17	8,00 ± 1,16	8,79 ± 1,18	2	B; E; A
6	Bisser	Haskovo	NM	wet meadow	38.50	0,31 ± 0,09	2,08 ± 0,44	2,39 ± 0,50	2	B; A
7	Dolnata ova	Haskovo	PL	wet meadow	38	1,57 ± 0,49	2,38 ± 0,96	3,95 ± 1,09	1	B; A
8	Lozenski pat I	Haskovo	PL	wet meadow	48	0,62 ± 0,23	3,14 ± 0,84	3,76 ± 0,82	2	B; A
9	Lozenski pat II	Haskovo	PL	wet meadow		0,37 ± 0,17	6,57 ± 0,99	6,39 ± 1,00	2	B; A
10	Kalinata	Burgas	PL	longos	20	0,98 ± 0,27	3,35 ± 0,54	4,33 ± 0,69	1	E
11	Blatoto	Burgas	PL	wet meadow	29	-	-	-	1	-
12	Petkaki	Burgas	unprotected	longos	0,50	-	-	-	1	-
13	Vesselie	Burgas	unprotected	longos	0,80	-	-	-	1	-
14	Arkoutino	Burgas	BZR	longos	0,25	-	-	-	1	C; D
15	Karaagach	Burgas	unprotected	longos	0,40	-	-	-	1	-
16	Chairite	Burgas	PL	wet meadow	2	-	-	-	1	E; A
17	Baltata	Varna	MR	longos	50	-	-	-	1	B; D; E

Category of the Protected area: PL - Protected Locality, NM - Natural Monument, BZR - Buffer zone of Reserve, MR - Maintained Reserve.
Vitality and Fertility: 1 - vigorous plants, commonly completing life cycle; 2 - vegetative propagation, little sexual reproduction; 3 - feeble, little reproduction.
Human impact: A - haymaking and grazing; B - draining of inundated and marshy terrains; C - incorrect afforestation practice; D - urbanization and assimilation of terrains for development of tourism infrastructure; E - incorrect gathering and overutilization of resources.

phenology, reproductive biology (Ilieva & Moynova 1969), phenodynamics and determination of Gal synthesis (Astadjov & Stancheva 1980; Stefanov 1990), agrotechniques (Ilieva 1967; Astadjov & al. 1980), etc. were carried out in 1961-1990 period.

IN VIVO CULTIVATION: As a result of mass selection a high-productive variety Snejinka was bred. Complete requirements for cultivation of summer snowflake were adopted in 1980 and first plantation was made near the village of Primorsko (Stefanov 1990). Method of clonal multiplication was developed (Tasheva 1993).

IN VITRO CULTIVATION: First experiments for application of *in vitro* methods on *L. aestivum* demonstrated that it is a recalcitrant object (Atanasov & Kikindonov 1972). Comparative study on morphogenetic potential showed that it varied among different populations and heavy contamination was an obstacle for micropropagation (Tchavdarov & al. 1984). Well developed plantlets of *L. aestivum* with normal diploid chromosome number have been successfully obtained by direct organogenesis from different vegetative organs (Fig. 1) (Stanilova 1991).

Since the *ex situ* practices need a lot of funds and special equipment within the experimental work, the economic processes and restititution of lands in Bulgaria in last 10 years impeded development of such initiatives and industrial transfer of results achieved.

A new strategy for protection of *L. aestivum* populations applying *ex situ* conservation methods was developed within the research project *Alternative approaches of bioproduction of alkaloids and active substances from Bulgarian rare and threatened medicinal plants* (NATO SfP-974453-Bioproduction) (2000-2005). The overall objective of the Project is to develop new or to improve existing *in vitro* and *in vivo* practices concerning *L. aestivum* as a main object. The major tasks of the Project are:

GERMPLASM GATHERING FROM WILD POPULATIONS - Seventeen natural gal-type populations of *L. aestivum* were assessed for their current status in 2001 and evaluated according possibilities to be used as donors of germplasm (Table 1, Fig. 1). Seven of them were selected for large-scale screening of clones by Gal content and bulbs and seeds were collected as genetic material for *ex situ* conservation.

FIELD CULTIVATION AND SELECTION - Methods for improvement of existing field cultivation practice were envisaged to be carried out: rapid bulb multiplication, breeding of high-productive clones. *L. aestivum* individuals of different origin will be maintained in *ex situ* collection for*in vivo* and *in vitro* experiments as well as for further propagation and *in situ* recultivation.

IN VITRO CULTIVATION - Different *in vitro* techniques were planned to be developed so to ensure effective micropropagation of the species. Gal producing shoot-clump liquid cultures and cell suspensions will be obtained.

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