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The geographic isolation of *Leucojum aestivum* populations leads to divergation of alkaloid biosynthesis

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ABSTRACT

Leucojum aestivum, an industrial source of the acetylcholinesterase inhibitor galanthamine, shows a great chemodiversity in its alkaloid synthesis. Samples from various geographically distinct populations from Bulgaria and the Balearic Islands were studied by GC–MS. The alkaloid pattern of the plants of *L. aestivum* (subsp. *pulchellum*) from the Balearic Islands were dominated by crinine type compounds. Populations of homolycorine chemotype were distributed along the Danube river in the north part of Bulgaria, which is separated from the south part by the Balkan mountains. Populations with high accumulation of lycorine were found in East Bulgaria near the sea coast, while the south populations were dominated by galanthamine type synthesis. The average of the galanthamine content was found to vary from 0.003 to 0.08% (referred to dry weight) in the north, and up to 0.42% in the southern Bulgarian populations. Some individuals showed up to 0.65% galanthamine. The galanthamine content of the plants from the Balearic island was 0.1% of DW. The galanthamine percentage in the total alkaloid mixture ranged from 0.2 to 95% of the total alkaloids. Our study demonstrated that the geographic isolation of the populations of *L. aestivum* has led to divergation in the alkaloid biosynthesis and consequently to the occurrence of different chemotypes. This chemodiversity in both alkaloid patterns and galanthamine content provides an opportunity for further selection work toward a galanthamine-rich crop, on the one hand, and makes the species an excellent biological system for molecular studies leading to further improvement of the galanthamine production, which is a valuable alkaloid used in medicine for the treatment of Alzheimer's disease.

1. Introduction

Leucojum aestivum L. (Amaryllidaceae), known as summer snowflake, is a threatened plant species that is currently used as a commercial source of galanthamine in East Europe. It is gathered from the natural habitats for industrial purposes which causes increasing problems with depletion of the wild populations. Galanthamine, a reversible acetylcholinesterase (AChE) inhibitor, is marketed as a hidrobromide salt under the name of Razadine® (formerly Reminyl®) for the treatment of

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Alzheimer's disease (Thomsen et al., 1991). According to data presented by the Alzheimer's Association (2010) the prevalence of Alzheimer's disease will quadruple by 2050. Despite that chemical synthesis of galanthamine is achieved (Marco-Contelles et al., 2006) plants still are used as an industrial source of this valuable natural product. Currently it is obtained also from *Narcissus pseudonarcissus* cv. Carlton, *Lycoris radiata*, and *Ungernia victoris* (Berkov et al., 2009a,b). *In vitro* cultures from *Narcissus confusus* and *L. aestivum* have been initiated for alternative production of galanthamine (Codina, 2002; Pavlov et al., 2007; Georgiev et al., 2009).

Existence of chemodiversity in distinct geographical locations is well documented for plants producing terpenes (Loreto et al., 2009) alkaloids (Wink et al., 1995; Macel et al., 2004), and phenolic compounds (Nyman and Julkunen-Tiitto, 2005). In a previous work on TLC analysis of the alkaloids from *L. aestivum*, intraspecies chemodiversity and variable galanthamine content was reported (Stefanov, 1990). Recent GC–MS analysis of dormant bulbs from this species revealed variable alkaloid patterns (Georgieva et al., 2007). For industrial purposes, however, the galanthamine content in the leaves and the galanthamine percentage in the total alkaloid mixture determine the value of the plant raw material. Generally, the galanthamine content of the plant raw material from *L. aestivum* collected from different wild populations and used for industrial extraction is *ca.* 0.1–0.2% of DW (personal communication). The chemodiversity of galanthamine producing species such as *L. aestivum*, provides an opportunity for selecting valuable genotypes to initiate both galanthamine-rich crops and *in vitro* cultures for industrial extraction. Chemodiversity has been also found for other amaryllidaceous plants like *Galanthus nivalis* and *Galanthus elwesii* (Berkov et al., 2011), which resulted in the isolation of a number of new alkaloids (Berkov et al., 2007, 2009). The reasons determining the chemodiversity and driving forces for the establishment of a specific chemotype in a specific location are still scanty studied in the family Amaryllidaceae.

As part of our ongoing studies on Amaryllidaceae plants, we herein report on the geographical chemodiversity in the alkaloid patterns and galanthamine content of *L. aestivum* plants collected from different locations in Bulgaria, as well as of *L. aestivum*, subsp. *pulchelum,* a species endemic to the Balearic Islands.

2. Materials and methods

2.1. Plant material

Samples were collected from 31 out of about 33 known populations of *L. aestivum* known in Bulgaria. Aerial parts from 10 to 40 individuals (depending on population size) from each sampling location of *L. aestivum* were randomly collected at the flowering stage in May 2009, and in 2010 for population 6 (Table 1). One-two bulbs per each population were also collected for chemical analysis. The samples were dried at 60 °C and then powdered in a mill. A voucher specimen (SOM-Co 1135) of the species was deposited at the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Bulgaria. Plants of *L. aestivum* subsp. *pulchellum* at the flowering stage were kindly supplied by Dr. David Bertran from the Botanical Garden of Barcelona. About 10 plants from each Bulgarian population were planted in an experimental field of the AgroBioInstitute, and leaf samples were collected at the flowering stage in the spring of 2011.

2.2. Alkaloid extraction

Dry material (100 mg) of from each individual plant was extracted for 12 h with 5 ml MeOH containing 50 μg of codeine as the internal standard (IS). Then aliquots of 4 ml from the extracts were evaporated to dryness. The dry extracts were dissolved in 3 ml of 1% H_2SO_4 and the neutral compounds removed with chloroform (3 × 3 ml). After basification of the extracts with

Table 1
Population codes, locations, habitats and river systems of *L. aestivum* plants collected in Bulgaria.

Popula-tion code	Locations	Habitat[a]	River system	Popula-tion code	Locations	Habitat[a]	River system
1	Antimovo	Wm	Danube	17	Lozenets	Ff	Karaagach
2	Archar	Wm	Danube	18	Jasna Poljana	Wm, Fl	Djavolska
3	Dobri dol	Wm	Danube	19	Blatets	Wm	Tundza
4	Orsoja	Wm	Danube	20	Jambol	Wm	Tundza
5	Belene island	Wm	Danube	21	Palauzovo	Wm, Ff	Tundza
6	Tutracan	Ff	Danube	22	Konevets	Wm	Tundza
7	Osmar	Wm	Kamchija	23	Elchovo	Wm	Tundza
8	Kochovo	Wm,	Kamchija	24	Ihtiman	Wm, –	Maritza
9	Novo Orjachovo	Fl	Kamchija	25	Chernozem	Wm, Ff	Maritza
10	Kranevo	Fl	Batova	26	Gradina	Ff	Maritza
11	Goritsa	Wm	Hadjiiska	27	Karadjalovo	Wm	Maritza
12	Kosharitsa	Wm	Hadjiiska	28	Ljubimets	Wm	Maritza
13	Prisad	Wm, Ml	Fakiiska	29	Biser	Ml	Maritza
14	Sozopol	Wm	–	30	Svilengrad 2	Wm	Maritza
15	Arkutino	Fl	Ropotsmo	31	Svilengrad 1	Wm	Matitza
16	Veselie	Fl	Ropotamo				

[a] Habitats: Forest (Ff-fluvial, Fl-longos), Wet meadow (Wm), and Marshland (Ml).