SfP-BIOPRODUCTION

SfP: 974453

Title: Alternative Approaches of Bioproduction of Alkaloids and Active Substances from Bulgarian Rare and Threatened Medicinal Plants

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Approval Date: 1 December 2000 Effective Starting Date: 15 February 2001
Duration: 4.5 years; expected completion by mid August 2005

NATO Budget: 371,840 EUR

Information about the SfP Project through Internet:

- home page of the project not yet established
- one page information available in Bulgarian via the on-line version of the journal "Bulgarian soldier": http://bgsoldier.eamci.bg/scripts/isapivwb.dll/doc?THEMEID=12232

Major Objectives

To provide the pharmaceutical industry with alternative technologies for biomass production of *Leucojum aestivum*, a rare and threatened plant species rich in alkaloid active against nervous diseases included AD, thus contributing to preservation of the natural plant populations.

- ✓ To evaluate the current status of the Bulgarian populations of *L. aestivum*
- ✓ To constitute representative germplasm collection from wild populations
- ✓ To evaluate snowflake agronomic and biochemical performances of *L. aestivum* under field conditions
- ✓ To characterize soil composition of diverse natural *L. aestivum* populations
- ✓ To evaluate, select and multiply clones with high contents of biologically active substances
- ✓ To identify suitable biological material for *in vitro* experiments, to develop efficient *in vitro* cell cultures and micropropagation
- ✓ To characterize alkaloid production *in vitro* and *in vivo*
- ✓ To develop lab scale *in vitro* cultures producing biologically active substances

Overview of Achievements since the Start of the Project until 31 October 2003

- ✓ A new Biotechnological Lab of Medicinal Plants, Institute of Botany, Sofia, has been installed, after major renovation funded by both BAS and the end-user and with equipment funded by NATO; Field plot and facilities were reorganized;
- ✓ Ecological assessment of 27 Bulgarian natural populations of *L. aestivum* (21 Gal-, 1 Lyc- and 5 Lycorenine-type) has been performed. The task leader (Institute of Botany, Sofia, Bulgaria) was mandated by the Bulgarian Ministry of Environment and Waters to expertise the regional inspections, which are locally in charge of the monitoring of *Leucojum* populations, on 2 aspects: resource assessment and determination of gathering quota (2001 and 2003).
- ✓ Plants and bulbs (3,500), seeds (441 progenies), 120 kg fresh herbage and over 300 model individuals of the *Leucojum* populations were collected in 2001 to 2003 for the purpose of the Project, in agreement with local authorities in charge of natural populations protection; Soil samples were collected from *Leucojum* populations for further analysis;
- ✓ A large Database on current diversity of Bulgarian *L. aestivum* populations and habitats (BLDB) has been developed; Texts and figures concerning all existing information for the species are compiled in different items; Development of different outputs is in process;
- ✓ Germplasm nursery has been constituted; Method for rapid bulb multiplication of *Leucojum* is under development;
- ✓ Soil control samples (30 from 9 natural populations) and 39 soil samples taken under plants were analyzed according to 37 soil parameters; Negative statistically significant correlations between Gal content and 7 parameters were proved;
- ✓ To assess effect of culture conditions on fresh mass productivity and Gal content (in 2003), over 300 model individuals from 10 populations were compared to 200 model individuals from the experiment field plot (transferred from the same populations in 2001). In general fresh mass productivity of cultivated plants is higher while their Gal content is lower then these of plants taken from wild, however Gal productivity of cultivated plants is valuable. Plant clusters screened for Gal content in 2001 and transferred to the experimental field were tested for their Gal-content alteration

after 2-year cultivation. In general the decrease of Gal content is statistically significant but it is important that the richest clusters in richest populations still remain rich.

- ✓ First selection of valuable individuals was made in 2003 based on their Gal yield.
- ✓ Callus formation was obtained from bulbs and from fruits, after an intensive study of culture conditions (growth regulators, environment, type of explants); Successful sub-cultivation has ensured formation of more friable callus but this step needs further improvement;
- ✓ Trial experiments in 3L-Lab Bioreactor were performed with already available cell suspensions from other plant species; The system is ready for experiments with *in vitro* culture of *L. aestivum;*
- ✓ First cultures of suspended cells and small aggregates of *L. aestivum* were obtained.
- ✓ Shoot clumps were obtained by *in vitro* micropropagation on bulb, stem, leaf and fruit segments; First bulblets have been rooted and transferred to greenhouse conditions, then to open air conditions;
- ✓ Karyological techniques using root tips are being set up for *L. aestivum*; 15 natural populations have been investigated for their chromosome number and karyotype structure; First *in vitro* obtained plantlets showed no substantial changes in chromosome number and chromosome structure.
- ✓ Analytical determination of Gal and related alkaloids by TLC has been developed for fast screening purposes; Protocols were elaborated for Gal extraction and determination with HPLC: in dry leaves (PDA detector) and in *in vitro* obtained calli (more sensible SF detector); CPC has been adapted for Gal purification;
- ✓ Lycorine was isolated in order to be used as standard. Isolation of other 5 related alkaloids is in progress.
- ✓ Comparative assessment of 10 populations (7 Gal-, 1 Lyc- and 2 Lycorenine-type) according Gal and Lyc content was carried out; They exhibit different patterns which could be interesting to connect with genetic variability;
- ✓ Routine HPLC analyses of different *in vitro* cultures (callus, shoot clumps) for Gal and Lyc content began in 2003; Most of samples contain Gal, however several other peaks could be also distinguished.
- ✓ Training of 3 young scientists was performed in France.

Payments through NATO Project Funds: 322,894 EUR

Milestones for the next six Months

- ✓ Completion of the BLDB: data from the investigations within the project;
- ✓ Monitoring of *L. aestivum* populations will continue;
- ✓ Recovery of bulblets obtained by twin-scaling will be estimated; Second-year selection of promising individuals will be done according main breeding characteristics;
- ✓ Optimization of callogenesis; Adaptation of the *L. aestivum* callus and shoot culture to submerged cultivation; Improvement of density and alkaloid content of cell suspension;
- ✓ Micropropagation; Improvement of sterilization; Multiplying of *in vitro* bulblets by cycle-procedure from plants of different populations from our field *in vitro* collection; Investigation of shoot growth in liquid medium; Study of influence of growth regulators on Gal synthesis; Study on rooting and acclimatization;
- ✓ DNA estimation of *L. aestivum* will begin using the MicroImage software;
- ✓ Routine HPLC analyses will continue for *in vitro* cultures; Related alkaloids obtained by CPC will be identified;
- ✓ Training of 1 young scientist from Partner 1 will be performed by Partner 4 on somatic embryogenesis;

Implementation of results

Results are not yet applicable at this stage. Our end-user (Sopharma Ltd., Sofia, Bulgaria) demonstrated their interest in Project with co-funding of setting up of the new BLMP. Sopharma was supplied with the first lyophilized *in vitro* callus lines and analyzed them. Renewal of the facilities and organization of Laboratory & Pilot Installation of Plant Biotechnology in NIHFI (acquired recently by Sopharma) with the aim to ensure the implementation of the project results is under discussion.

NATO Consultant:

Pr. Michael Heinrich,
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Additional Collaborating institutions

None.

Abbreviations:

AD	:	Alzheimer Dementia
BAS	:	Bulgarian Academy of Sciences
BLDB	:	Bulgarian *Leucojum aestivum* DataBase
BLMP	:	Biotechnological Laboratory of Medicinal Plants
CPC	:	Centrifugal Partition Chromatography
HPLC	:	High Performance Liquid Chromatography
Gal	:	Galanthamine
LSDR	:	Laboratoire de stress, défenses et reproduction des plantes
NIHFI	:	Chemical Pharmaceutical Research Institute-Co

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OPTIMA : Organization of Phyto Taxonomic Investigations of the Mediterranean Area
TLC : Thin-Layer Chromatography