ВЪЗМОЖНОСТ ЗА *EX SITU* ОПАЗВАНЕ НА ЗАСТРАШЕНА ПОПУЛАЦИЯ ОТ ХРАСТОВИДЕН ПЕЛИН (*ARTEMISIA CHAMAEMELIFOLIA* VILL., *ASTERACEAE*) ЧРЕЗ МЕТОДИТЕ НА *IN VITRO* КУЛТИВИРАНЕТО

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EX SITU CONSERVATION OF A THREATENED POPULATION OF *ARTEMISIA CHAMAEMELIFOLIA* VILL. (*ASTERACEAE)* THROUGH THE METHODS OF *IN VITRO* CULTIVATION

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ABSTRACT

Artemisia chamaemelifolia Vill. (*Asteraceae*) is an aromatic perennial shrub with a status of a critically endangered species for the territory of Bulgaria. It is a glacial relict growing in grassy and stony karst areas in the mountains of Southern Europe: Southwestern Alps, Pyrenees, Cordillera Cantabrica (1) and the Temperate Asia: North Caucasus, Transcaucasia (Azerbaijan, Armenia, and Georgia), Asiatic Turkey and Iran (2). Its Bulgarian population is localized only in a restricted area in the Ponor mountain (part of Northwestern Stara planina mountain range) and it is subjected to the following threats: aridization and destruction of habitats by tourists (trampling and drying) (2). Furthermore, the small Bulgarian population, the low *in vivo* reproductive potential and the very specific habitat characteristics worsen the status of this species (3). The *in vitro* cultures can be successfully applied for the *ex situ* conservation of the genetic fund of endangered plant species can (4; 5). At the Plant Biotechnology laboratory at the Plant Physiology department of the Biology Faculty - Sofia University "St.Climent Ohridski" we initiated *in vitro* culture of *A. chamaemelifolia*. There was developed a protocol for seed sterilization, micropropagation and sustainable long-term *in vitro* cultivation of *A. chamaemelifolia* on plain MS medium supplied with Gambourg B5 vitamins (6; 7). We are working on a protocol for adaptation of the *in vitro* cultured plants to greenhouse conditions that would aid the reintroduction into the natural habitats of *A. chamaemelifolia*.

Key words: Artemisia, ex situ conservation, in vitro cultivation.

INTRODUCTION

The *ex situ* conservation is a method for protection of threatened plant or animal species outside of their natural areal by removal of part of the population from endangered habitats and their localization in a new place, which can be in the wild or within human care (natural reserves, zoos, botanical gardens, laboratories, etc.). For a maximum conservational importance, the *ex situ* samples should be genetically representative – with the allelic diversity and the evolutionary potential of the original natural populations.

The storage of germplasm from rare and threatened plant species in laboratory *(in vitro*) is suitable for long-term conservation of vegetatively reproduced species (4; 5).

Ex situ conservation by vegetal biotechnologies requires:

1. The establishment and improvement of the methods for collection, disinfection, regeneration and conservation of the plant species;

2. The genetic stability evaluation of the plant material preserved in gene banks and collections (8).

The micropropagation of plants allows growth in controlled environment on nutrient medium in closed sterile containers. The process can be carried through the whole year regardless of the season and the climatic conditions. This is an essential method for *ex situ* conservation of rare and threatened plant species, species with reduced populations or low fertility, and for fast propagation of valuable medicinal plants, as well.

Artemisia chamaemelifolia Vill. (*Asteraceae*) is an aromatic perennial shrub with a status of a critically endangered species for the territory of Bulgaria. It is a glacial relict growing in grassy and stony karst areas in the mountains of Southern Europe: Southwestern Alps, Pyrenees, Cordillera Cantabrica (1) and the Temperate Asia: North Caucasus, Transcaucasia (Azerbaijan, Armenia, and Georgia), Asiatic Turkey and Iran (2). Its Bulgarian population is localized only in a restricted area in the Ponor mountain (part of Northwestern Stara planina mountain range) and it is subjected to the following threats: aridization and destruction of habitats by tourists (trampling and drying) (2). Furthermore, the small Bulgarian population, the low *in vivo* reproductive potential and the very specific habitat characteristics worsen the status of this species (3). The *in vitro* cultures can be successfully applied for the *ex situ* conservation of the genetic fund of endangered plant species can (4; 5).

MATERIAL AND METHODS:

Ripe dry seeds of *Artemisia chamaemelifolia* Vill., gathered in 2009 from the locality of Breze village in the Ponor mountain, altitude of about 1560 m.

Seed Treatment:

1. Cold stratification - at 4°, in dark, for 4, 8 and 16 months.
2. Seed sterilization:
   a) Pre-sterilization in 70% ethanol for 1-2 minutes.
   b) Sterilization in 100% Domestos® (commercial preparation of sodium hypochlorite) with 4 - 5 % NaOCl (4,8 g/100 ml) - dipping for 20 minutes.
   c) Triple soaking and rinsing with sterilized distilled water.

Germination experiment:

1st Experiment - The seeds were planted in sterilized water agar (8 g/l), poured and autoclaved in test tubes beforehand, and they were left in dark, at room temperature (18 - 21°C).

2nd Experiment - The seeds were planted in half-strength MS medium, with 8 g/l agar and pH = 7,75 - 7,8, poured and autoclaved in test tubes beforehand, and they were left in dark, at room temperature (18 - 21°C).

3rd Experiment - 50 seeds planted in water agar and 50 seeds planted in half-strength MS medium, with 8 g/l agar and pH = 7,75 - 7,8, poured and autoclaved in test tubes beforehand, and they were left in dark, at room temperature (18 - 21°C).

***In vitro* cultivation:**

The seedlings were transferred on a plant regeneration medium after they began to photosynthesize and produce more than two leaves. We used plain MS medium supplied with Gambourg vitamins, 30 g/l carbon source (sucrose), 8 g/l agar as a gelling agent, and pH = 7,75 - 7,8, at 16/8 h light period and 23±1°C. The transfer on fresh medium is carried every 30 days (1-month passage).

***Ex vitro* adaptation:**

Firstly, after the 5th passage on fresh medium, 10 of the highest and best regenerated plants were left in their old medium to stimulate the better formation of a stronger root system. Then we took them out of the *in vitro* containers, cleaned and measured them, and planted them in pots covered with plastic transparent cups, perforated for air flow. The pots were layered in advance with a drainage layer of small stones, a middle layer of sand and an upper layer of sand-peat mix thus

imitating the habitat soil conditions. The pots are kept in the green house of the Biology Faculty, at 20 C, in natural light. The procedure is performed repeatedly every 2-3 months for gathering enough material for *ex vitro* adaptation.

RESULTS AND DISCUSSION:

One out of 11 available and usable planted seeds germinated in 5 weeks time during the 1st germination. The 2nd germination turned out unsuccessful since not a single seed germinated. This suggests that the plain water agar is a suitable medium for germination, whereas some of the half-strength MS medium factors might prevent the seeds from germination - i.e. the pH. The 3rd germination ended with 18% germinated seeds on water agar, and 12% germinated seeds on half-strength MS medium.

So far, we've had a successful stable *in vitro* propagation of *Artemisia chamaemelifolia* Vill. One seedling was enough to set up an abundant culture that has multiplicated without contaminations. The regenerants are growing with well developed leaf biomass and root system. The MS type of medium combined with Gambourg vitamins is suggested by several publications to be the most suitable for the regeneration of whole plants of *Artemisia* species (6; 7). It does not contain growth regulators that might contribute to the genetic variations of the culture and lower the opportunity for reintroduction of the in vitro regenerants into their original habitat.

It took four months for the best regenerated *in vitro* plants to become physically strong and suitable for the adaptation - with strong roots and well developed leaves and stems. The first 10 plants were planted in pots and since then they are developing steadily, without contaminations. The outcome after the 1st planting is 20% survival. The 2nd planting resulted in 80% survival. The 3rd planting is in process of early adaptation (3rd week).

CONCLUSION:

Despite of the difficult germination, *in vitro* cultivation of *Artemisia chamaemelifolia* Vill. is easily carried out to obtain a rich culture. The species features offer lots of options for conducting new experiments - usage of different techniques for breaking the seed dormancy, experimental design for different nutrient media aiming at faster and better whole-plant regeneration. The short period of *ex vitro* adaptation is promising good outcome of the experiment - the plantlets are stable and well developing allowing further reintroduction into their native habitats.

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