

## EX SITU COLLECTION OF MODEL RESURRECTION PLANT *HABERLEA RHODOPENSIS* AS A PREREQUISITE FOR BIODIVERSITY AND CONSERVATION STUDIES

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### ABSTRACT

Resurrection plants are considered model species in studies that are focused on the improvement of the abiotic stress tolerance of crops. Belonging to different botanical families and living under various environments, these species possess one common feature- their vegetative tissues are able to withstand long periods of full desiccation and to recover rapidly upon re-watering. *Haberlea rhodopensis* is an endemic of the Balkan Peninsula with a well known desiccation tolerance and a subject of intensive studies in the recent years. Here, we present the establishment of an *ex situ* collection from the 12 main localities where the species could be found in Bulgaria. A successful, simple and uniform protocol for *in vitro* propagation for plants from all localities has been developed. Thus, we are able to perform intensive biodiversity studies, to propagate routinely large amounts of true-to-type plant material for various purposes and to reintroduce *Haberlea* in the nature if the respective localities are put under environmental and/or human challenges.

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**Keywords:** *Haberlea rhodopensis*, *ex situ* collection, *in vitro* propagation, direct organogenesis, true-to-type propagation; biodiversity

**Abbreviations:** WPM: Woody Plant Medium; GPS: Global Positioning System; HgCl<sub>2</sub>: Mercuric chloride

### Introduction

It became obvious in the recent years that changing environment will have an increasing negative effect on global agriculture. Despite the enormous efforts and technological progress, it is now very difficult to improve crops' adaptability to harsh environment, to secure sustainable development and increased productivity. The various strategies to survive desiccation should be considered an important prerequisite to secure yields and in this respect the so-called resurrection plants are excellent model systems because of their unique tolerance (7). These plant species, about 300 in number, belong to different botanical families and live under various environments. Their only common feature is the ability of their vegetative tissues to withstand long periods of full desiccation and to recover rapidly upon re-watering (3, 16).

Bulgaria is among the few countries in Europe where two resurrection plants species of the Gesneriaceae- *Haberlea rhodopensis* Friv and *Ramonda serbica* Pancic live in natural habitats (1, 12). *H. rhodopensis* was discovered for the scientific society in the middle of 19<sup>th</sup> century (15) and about a century later its resurrection behavior was documented (8). Various parameters of the reaction to desiccation and recovery are under extensive studies in the last twenty years (7). As a

rule, these investigations were performed with samples taken directly from nature or botanical gardens.

After the first successful protocol for efficient *in vitro* propagation of *Haberlea rhodopensis* (6) other groups announced their efforts to propagate *in vitro* *Haberlea* (5) and *Ramonda serbica* (8, 14). Apart from its unique desiccation tolerance, there is an increasing focus on *H. rhodopensis* as a species with potential for multipurpose uses (2, 4, 7, 10, 13). Additional interest to study the biodiversity and plasticity of *Haberlea* is related to the fact that at the beginning of 20<sup>th</sup> century a new related species (*H. feridnandii-coburgii* Urum.) has been proclaimed (17).

The aim of the present study was to develop an *ex situ* collection from the main *Haberlea* localities in the country, including the one where the potential origin of *H. feridnandii-coburgii* was announced and to establish efficient and if possible, universal *in vitro* propagation system.

### Materials and Methods

#### Expeditions

Localities were visited in accordance to the information for the distribution of the studied species based on the available literature data and specimens deposited in the Herbaria of Sofia University (SO) and Institute of Botany at Bulgarian Academy of Sciences (SOM), and the Herbarium at the Agricultural University, Plovdiv (SOA). Samples were collected between April and August 2008 and voucher specimens were deposited in the Herbarium of Sofia University St. Kliment Ohridski (SO). For each locality coordinates and altitude were evaluated with a GPS, model V, GARMIN, 2003, part number-190-00204-11. The localities were mapped and photographed.

TABLE 1

Geographic details of *Haberlea rhodopensis* accessions from Bulgaria

LOCALITIES	Specimen	Latitude (N)	Longitude (E)	Altitude range (m a.s.l.)
<b>Rodopi mountains</b>				
<b>Eastern Rodopi</b>				
Dyavolskiya most, Ardino	SO 105795	41°37.230'	25°06.892'	416
Studen kladenetz	SO 105796	41°36.452'	25°38.810'	153
<b>Western Rodopi</b>				
Asenova krepost	SO 105793	41°59.290'	24°52.260'	410
Bachkovo	SO 105794	41°56.665'	24°51.433'	372
Devin	SO 105797	41°44.73'	24°22.453'	812
Trigrad Gorge	SO 105798	41°37.275'	24°22.943'	1474
Mihalkovo	SO 105799	41°50.894'	24°25.240'	550
Shirokolashka river	SO 105800	41°42.565'	24°27.206'	786
<b>Balkan mountains</b>				
Lovech	SO 105809	43°07.427'	24°43.579'	202
Malusha	SO 105801	42°44.989'	25°16.905'	1312
Plachkovci	SO 105802	42°45.964'	25°30.133'	1061
Byala reka, Kalofer	SO106162	42°39.791'	24°57.609'	1278

TABLE 2

Morphogenic response of explants taken from different *Haberlea* localities in Bulgaria

No.	LOCALITIES	Regeneration rate	First appearance of regenerant (days)
1	Asenova krepost	9.5±0.2	65
2	Bachkovo	12.0±0.1	45
3	Dyavolskiya most, Ardino	11.0±2.1	80
4	Studen kladenetz	12.9±0.2	70
5	Devin	8.7±0.1	90
6	Trigrad Gorge	7.9±0.1	60
7	Mihalkovo	9.3±0.1	60
8	Shirokolashka river	4.2±0.1	50
9	Lovech	5.5±0.1	80
10	Malusha peak	9.3±0.2	70
11	Plachkovci	10.2±0.1	70
12	Byala reka, Kalofer	33.0±0.1	45

Regeneration rate was estimated as % of explants with regenerants and is presented as mean±SE from four replications. The speed of morphogenic response is presented as period (in days) from culture initiation to the appearance of first regenerant

### **Ex situ collection with plants from the natural localities**

Several plants per locality were transferred to the greenhouse of Agrobiointitute to establish a source for explants for *in vitro* propagation.

### **In vitro cultures**

To initiate *in vitro* culture of *Haberlea* we used fresh fully developed young leaves as explant sources. Surface sterilization with 70% ethanol for 1 min, followed by 3-7 min treatment with 0.1% HgCl<sub>2</sub> and subsequent washing with sterile distilled water was tested for efficiency.

### **Morphogenic response**

At least 30 explants from plants of various localities were tested for regeneration response. The reaction was evaluated at 5 day intervals for a 3 month period. The experiments were repeated 4 times at every 3 months to avoid eventual seasonal response.

Culture initiation, direct organogenesis and culture transfers were performed on basic WPM (11). To achieve better plant growth and rooting we modified our procedure (6) by performing the last transfer of the plantlets in tubes with liquid medium and paper bridges.

### **Pot plants**

Well developed and rooted plantlets were moved to pots under greenhouse conditions.

## **Results and Discussion**

### **Expeditions and collection of plant material**

Samples were collected between April and August 2008 and voucher specimens were deposited in the Herbarium of Sofia University St. Kliment Ohridski (SO) (Table 1).

Since its very discovery in the mid 19<sup>th</sup> century *Haberlearhodopensis* was documented in Rhodopa and Stara planina (Balkan) mountains (15). Here, we present, we believe for the first time a list of the main *Haberlea*'s localities (Table 1), well-documented chorology, including the one near Lovech- the only one place where the putative *H. ferdinandii-coburgii* has been marked (17). The species shows remarkable plasticity (5, 12). In our case we found it at altitudes from 150 to almost 1500 m which could be a good clue for further biodiversity studies.

### **Establishment of ex situ collection**

#### **Regeneration in vitro**

As previously described (6), the first successful protocol for *in vitro* propagation of *H. rhodopensis* was initiated from seeds. The same method was recently applied in another group's attempt to establish collection of *Haberlea* (5). Here we present a different approach with the use of leaf explants. The idea was to shorten the process of culture initiation and to assure the full genotype identity of our cultures to the plants taken from the respective localities. Because of their hairy surface the *Haberlea* leaves are quite difficult for sterilization. We tested

several variants and the best appeared to be pretreatment for 30 sec with 70% ethanol followed by 4 min treatment with 0.1% HgCl<sub>2</sub>.

Further-on the sterile leaf segments were used as explants for culture initiation on standard WPM (11). Direct organogenesis occurred for 1.5-3 months in the cultures from various localities (Table 2, Fig. 1A, Fig. 1B). Modification of previous protocol (6) by introduction of liquid media and paper bridges at last stages of plant growth resulted in shortening the culture period, well developed root system and larger plantlets (Fig. 1C).

The use of a simple medium combination is widely accepted as the most reliable micropropagation procedure when true-to-type propagation is aimed. Omitting the addition of plant growth substances or other compounds we were able to evaluate the potential variation in morphogenic response of the plants from different localities. Interestingly, there were remarkable differences both in the rate of response and regeneration speed (Table 2). Explants taken from the localities Shiroka luka (No. 8) and Lovech (No. 9) were with lowest regeneration rate-about 5%, while most of the other localities showed about 10% morphogenic rate. The locality Kalofer (No. 12) was the only clear exception with very high regeneration rate (33%). Fastest response was found in plants taken from the localities Bachkovo (No. 2), Shiroka luka (No. 5) and Kalofer (No. 12) (about 45-50 days after culture initiation). It took more than 60 days for the explants taken from the other localities to initiate regeneration with slowest response in explants from Devin locality (No. 5).

Since there are no data available for similar studies on morphogenic response by other groups or with other resurrection type plant species, it is difficult to draw any conclusions for potential relationship between the locality origin and regeneration potential. Further biodiversity studies based on modern molecular approaches (7) could pit additional light on these interesting phenomena. The fact that the explants taken from the locality of special interest (Lovech No. 9), where more than a century ago a related species (*H. ferdinandii-coburgii*) (17) has been documented, have no significant difference in morphogenic response with most of the other localities could be an additional clue towards the confirmation of the idea that *Haberlea* in this region is not botanically different (15). Further molecular studies could prove this statement (7).

#### **Pot plants collection**

After the establishment of efficient *in vitro* propagation system, we were able to propagate routinely plants from all studied localities of *Haberlea*. When the respective stage of growth and rooting was reached, the plants were moved to pots under greenhouse conditions (Fig. 1D).

Micropropagation with the use of plant growth regulators and/or other substances as a rule raises questions about the potential risk for somaclonal variation or other deviations from the initial genotype. Although our regeneration procedure is

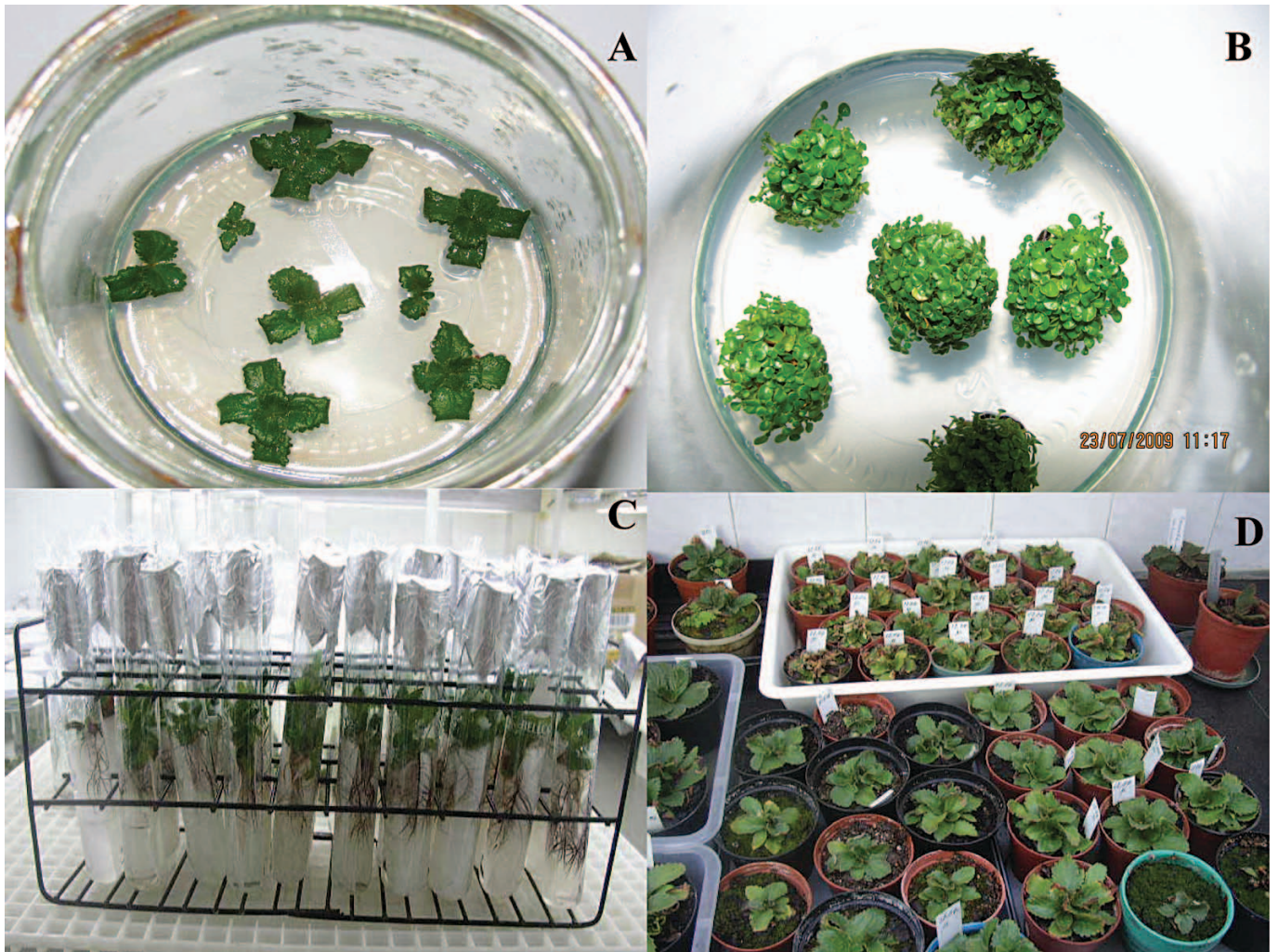


Fig. 1. Efficient and uniform *in vitro* propagation system of *Haberlea* from various localities

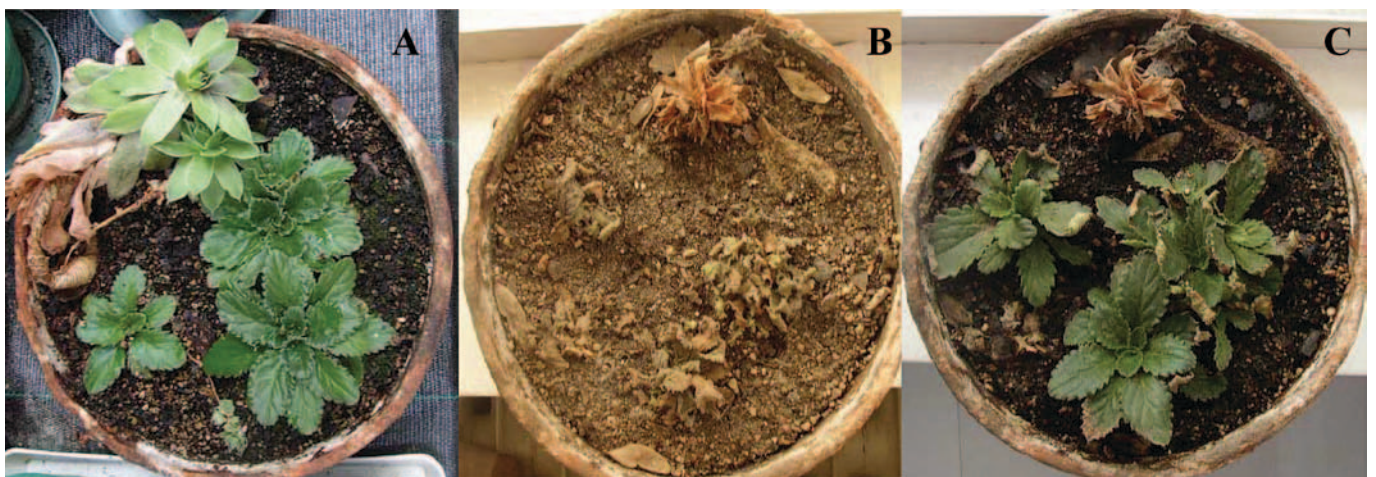


Fig. 2. Successful recovery of *in vitro* developed and potted *Haberlea* plants after drying for 2 months compared to non-resurrection type *Sepervivum* sp.

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strictly on basic culture medium we proved that our regenerants are true-to-type by testing their desiccation tolerance (Fig. 2). *In vitro* developed and potted *Haberlea* plants (Fig. 2A) were left without water for more than two months (Fig. 2B) and re-hydrated again. In less than 48 h they were able to recover fully compared to the completely desiccated control species *Sempervivum tectorum* L. (Fig. 2C).

## Conclusion

We were able to establish an *ex situ* collection of *Haberlea* plants from the 12 main localities of the species in Bulgaria. Using leaf explants we developed a successful, simple and uniform protocol for direct organogenesis and *in vitro* propagation for plants from all localities. Differences in morphogenic response between plants from various localities have been found that are an additional challenge for further molecular and biodiversity studies. The availability of a routine propagation system is of crucial importance to secure large amounts of true-to-type material of endangered and rare plants such as *Haberlea* for various purposes including reintroduction in the nature.

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