Breeding systems of *Haberlea rhodopensis* (*Gesneriaceae*), a Tertiary relict endemic to the Balkan Peninsula

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Abstract. This study presents the preliminary results on sexual reproduction of *Haberlea rhodopensis*, *Gesneriaceae* – a Tertiary relict and endemic species to the Balkan Peninsula. Our experiments have shown that *H. rhodopensis* is self-compatible, but not autogamous. Phenology of the populations, herkogamy and weak protrandry are mechanisms which favour the outcrossing in natural populations. Pollination success is characterized by high seed production. Seeds germinate readily, but grow extremely slowly and few of them survive to adult plants in culture and in natural populations.

Key words: breeding systems, floral mechanism, *Haberlea rhodopensis*, phenology, seed set

Introduction

Haberlea rhodopensis Friv. (*Gesneriaceae*) is a Tertiary relict and Balkan endemic. In Europe, there are only three genera in the family: *Haberlea* (in Bulgaria and Greece), *Ramonda* (Balkans and Iberian Peninsula) and the monotypical *Jankaea* (in Greece) (Ganchev 1950).

Haberlea rhodopensis is a medicinal plant, listed in the Bulgarian Medicinal Plants Act (2000). It is also an amazing resurrection plant; therefore, recently most investigations have focussed on its desiccation tolerance mechanisms and potential for multipurpose usage. Efforts at conservation of *Haberlea rhodopensis* are based mainly on *in vitro* cultures, *ex situ* collections and habitat exploring (Djiljanov & al. 2009). The plant propagates vegetatively and it is possible to cultivate it from seeds (Ganchev 1950; Djiljanov & al. 2005), but there are no studies on its breeding system.

The aim of our study was to throw light on the breeding systems of *Haberlea rhodopensis:* 1) to test outcrossing of the species and its mechanism; 2) to test seed germination and early stages of ontogenesis; 3) to compare native populations and plants grown *ex situ*.

Material and methods

We have used plant material collected from five natural localities in Central and Eastern Rhodopi Mts, Bulgaria and an *ex situ* collection from the Botanical Garden at Johannes-Gutenberg University of Mainz, Germany. Observations were conducted in 2008–2010 and 2012, during the flowering pe-

riod from April to July. For laboratory works, we used the Leitz Diaplan light microscope with fluorescence equipment, Binokular Leica MZ 16A, Fuchs-Rosenthal counting chamber, Philips XL 30 scanning electron microscope (SEM), and Leica Application Suite software and Image Tool MT 3.00 for photographs, pollen and ovule counting. The authors have followed the manufacturers' instructions and standard protocols.

Phenology. In the flowering period (April–July) of 2008–2010 and 2012, *in situ* (natural populations) and *ex situ* observations have been carried out at different altitudes and slope exposures. Life duration of free and bagged-flower plants was compared. For the purpose, two sites were chosen with different sun exposure, so as to trace out any differences in phenology, according to the autecological factors.

Breeding systems. In order to test autogamy, the protocols of Dafni (1992) were considered and consequently were bagged: a) a total of 123 flowers of 29 plants for spontaneous self pollination, b) 38 hand-selfed flowers from six plants, c) four hand-crossed flowers from four plants, and d) 10 untreated control flowers from three plants. Plant rosettes were transferred to the experimental plot of the Faculty of Pharmacy at different time of the year. To test ability of propagation by leaves, 10 leaves were put into a light soil mix. Ovaries and anthers were sampled from three different sampling sites: Beden village in 2008 (Central Rhodopi), river Borovitsa in 2010 (Eastern Rhodopi), Ustovo district in 2010 (Central Rhodopi), and the Botanical Garden of Mainz University, Germany in 2012. Pollen grains and ovules were counted and calculations were performed according to the standard methodology (Cruden 1977; Daphni 1992).

Floral mechanism. To investigate dichogamy and/or herkogamy, we have dissected and photographed flowers in different stages.

Stigma receptivity. To evaluate changes on the stigma surface indicating receptivity, we have collected stigmas from flowers in different phases of development, and photographed them with SEM.

Pollen tube growth. Six flowers in bud stage were bagged and hand-selfed. We sampled the stigmas 3, 4 and 20 hours after pollination and have observed the pollen tube growth under a fluorescence microscope.

Pollination success. Seed set per fruit of a random sample in plants from different sampling sites was recorded.

Seed germination and mycorrhiza. Fifty fresh seeds from the Ustovo district and the Botanical Garden of Mainz and 50 two-year old seeds from Yagodina and Mugla villages were placed in Petri dishes on wet filter paper, or directly in soil, without additional treatment. Three weeks later the two-year old seeds were scarificated. Squash microscopic preparations of roots were made following the standard method, so as to test the plant for mycorrhiza.

Results and discussion

Phenology. Anthesis is most prolonged in the natural populations (22 days) and the shortest in the shady population from the Botanical Garden (12 days). The exposed to sunshine *ex situ* population had an 18-days anthesis. In spite of the same altitude, the anthesis in the shady population of the Botanical Garden began a week later, but ended at the same time. Compared with the natural habitat at 800 m, anthesis in the *ex situ* population had begun a month earlier at about 100 m. A comparison of the results in the natural habitat for the years 2010 and 2012 has shown that in 2010 anthesis began on 15th May, and in 2012, after a very long and cold winter, it began on 24th May (Fig. 1). Life duration of a flower was prolonged between 7–10 days and no difference was traced out between free and bagged flowers. Observation also showed that the flowers in inflorescence of one individual were in different phases of flowering, as well as the individuals in a population.

On 24th August 2008, secondary flowering was observed in the area of Chudnite Mostove (Central Rhodopi, at 1500 m). The flowering buds were laid in autumn and were preserved in winter time.

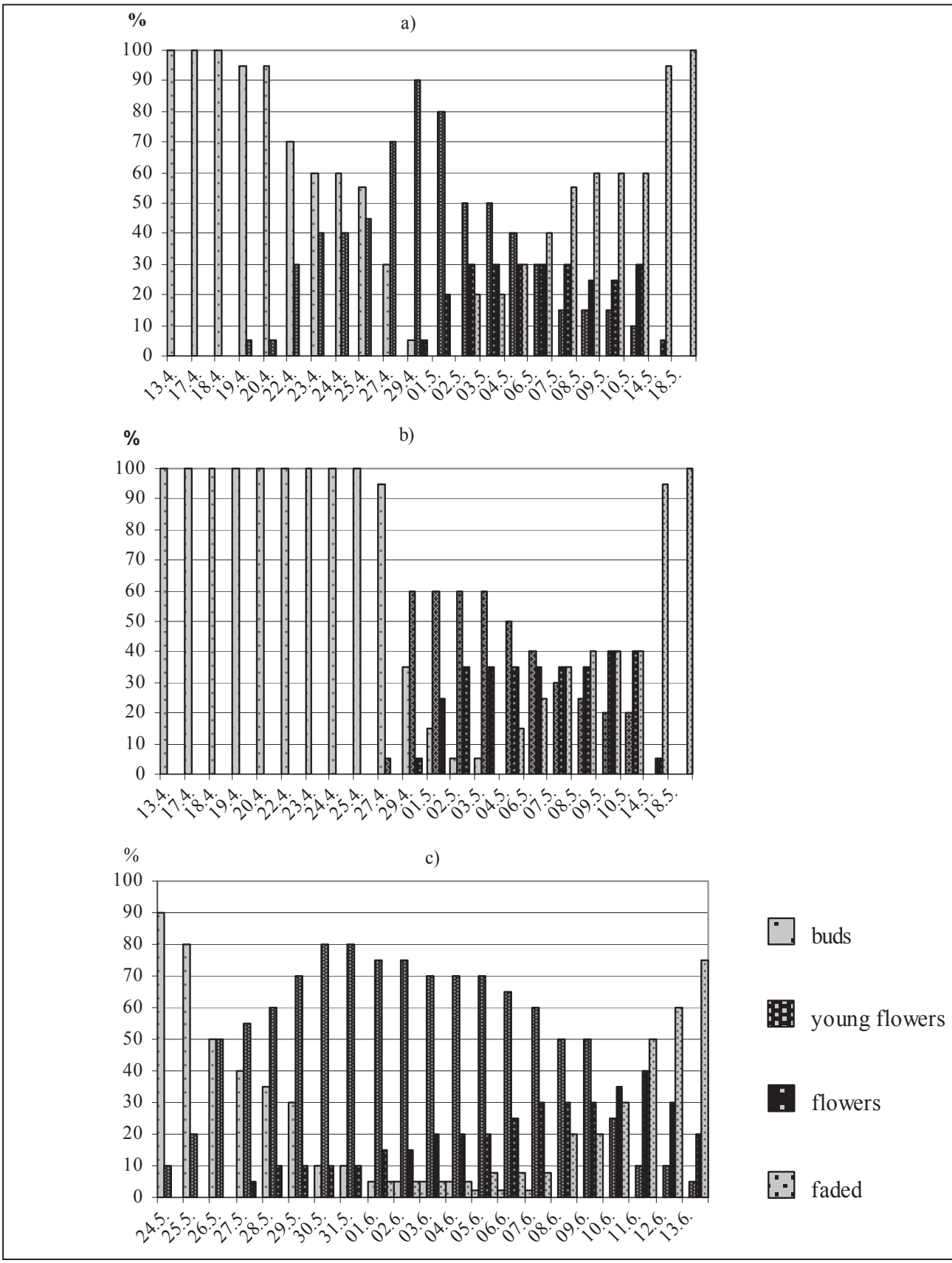

Fig. 1. Comparative phenology of three sites: **a)** population exposed to sunshine from the Botanical Garden of Mainz University, 100 m a.s.l.; **b)** population in shadow from the Botanical Garden of Mainz University; **c)** natural population in Ustovo district, Central Rhodopi , 800 m a.s.l.

Breeding system. *Haberlea rhodopensis* is a long-living perennial (Plate 1, Fig. 1). Plants of the white variety ('Virginalis') are grown in the UK since 1938, and still grow strongly in the course of 75 years. This corresponds to the reports concerning the age of plants from the sister species *Ramonda myconi*. Data on the accumulated individual growth in size (number of leaves and rosette diameter) suggest that under the current environmental conditions the time from germination to attainment of the minimum size for reproduction is *ca*. 70 years. Similarly, the estimated age of a plant with 11 leaves and rosette diameter of 12 cm (the median size of plants in all the regions) is 200–250 years (Dubreuil & al. 2008).

Haberlea rhodopensis has good ability for vegetative propagation (Plate 1, Fig. 1, Plate 2, Fig. 1). *In situ* the big plants shoot thin pale-yellow horizontal rhizomes, running under the moss around the plants, and bear small rosettes. Such rhizomes have never been seen in cultivated plants, however, it was certainly easy to divide the large plants and grow up many of the divisions *ex situ*. Even some *ex situ* propagation by leaf was possible: three of the leaves produced daughter rosettes, although they remained extremely small and did not develop into big plants. Plants transferred in spring and early summer, especially if taken with the moss cushion, were doing very well *ex situ* (Plate 1, Fig. 4, On the other hand, if the plants were transferred in the autumn they had zero ability to survive.

The flowers bear features of bee pollination syndrome (Plate 1, Fig. 2, Plate 2, Fig. 2,). The tested flowers did not self-pollinate spontaneously. None of the 123 bagged flowers tested for spontaneous self-pollination set seeds. There was no self incompatibility, as 16 flowers (50 %) of the hand-selfed ones produced seeds. The control hand-crossed four flowers (100 %) produced seeds, which have proved that bagging does not effect the process of pollination and fertilization. Eight of the ten untreated flowers set seeds (80 %).

An alternative method for testing the breeding system by calculation of P/O ratio corresponded partially to the field tests. Interestingly, different values of pollen-to-ovule ratio were recorded for the different sampling sites: Beden village 308:1, river Borovitsa 94:1, Ustovo district 141:1, and Mainz Botanical Garden 141:1. Following Cruden`s classification of the breeding system P/O ratio, varying in the populations between 94:1 and 308:1, indicates an obligate to facultative autogamy (Cruden 1977). If taken into account that pollination success is not dependent solely on pollen limitation, but is due to sex allocation, it could be maintained that the increased number of ovules is not just an autogamy indicator, but an attempt at ensuring a maximum seed set (Etcheverry & al. 2012).

Pollen tube growth. Our results show that self pollen germinates on the stigma of the same individual. Three hours after pollination, pollen germination is at its very beginning. After four hours the pollen tubes can be clearly distinguished and twenty hours after pollination the pollen tubes reach about 2–3 mm in the pistil (Plate 1, Fig. 6).

Floral mechanism. In our opinion, outcrossing was favoured under natural conditions: weak protandry was observed and strong herkogamy, as mechanisms which support outcrossing. In the bud stage, the anther length exceeded the length of the pistil and they were situated above the stigma (Plate 1, Figs 3, 5). In many cases, the extrorse pollen-shedding anthers opened in the bud stage, thus self pollen deposition on the stigma was possible, but at that time the stigma was not receptive.

Stigma receptivity. Changes in the stigma area indicating receptivity are visible on the microscopic pictures 2–3 days after the flower opening (Plate 2, Figs 5, 6). During bud development the pistil grows and, when the flower opens, the pistil length exceeds the length of anthers. Anthers remain fastened in pairs about 3 mm under the stigma level on the pistil.

Pollination success. Eighty percent of the randomly collected fruits for estimation of the free pollination success had seeds. Sixteen percent of the flowers had not been pollinated and four percent were predated. Seed set in a fruit of a random sample showed that on the average 70 % of the ovules per fruit have matured to seeds (Fig. 2).

Obviously, when self pollen reaches the receptive stigma, pollination starts and seeds are formed, but a pollen-transporting agent is needed. The relatively high fruiting success of the control flowers shows

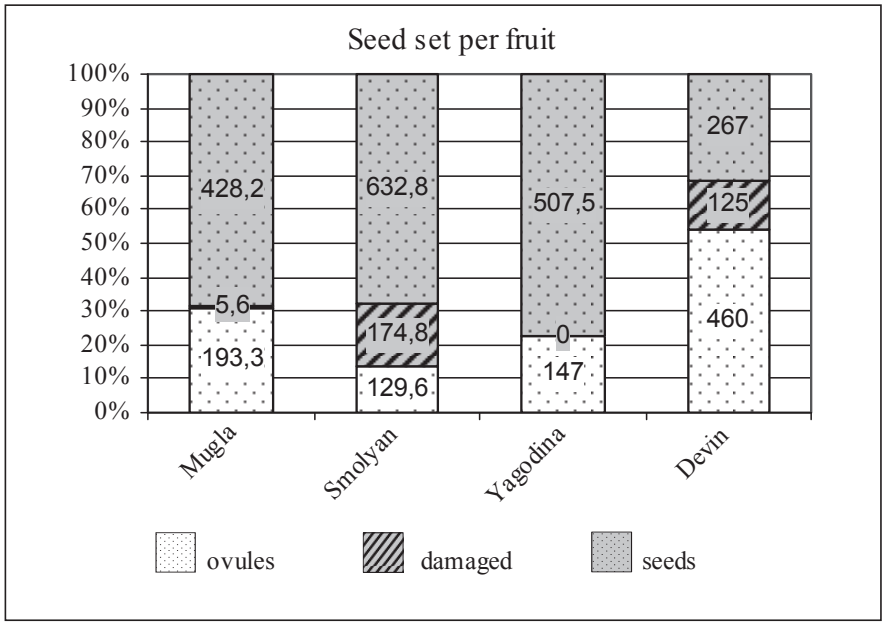

Fig. 2. Seed set per capsule of random samples from four different natural populations in the Central Rhodopi Mountains.

that pollination is efficient, but bagging experiments reveal that *Haberlea* is self-compatible, though not generally autogamous. Our results correspond to the data obtained for the related species *Jankaea heldreichii* (Vokou & al. 1990) in Greece, which also produces a high number of seeds per capsule and is not autogamous, as well as for *Ramonda myconi* on the Iberian Peninsula (Pico & Riba 2002).

Seed germination and mycorrhiza. The seeds of *H. rhodopensis* readily germinate after 7–12 days. Seedlings grow extremely slowly, reaching a length of less than 4 mm in five months as shown in Plate 2 Fig. 6. After five months, less than 1 % of the seeds survived. Two-year old seeds did not germinate, even after being scarificated for a week. The root microscope preparations clearly show the structures, which indicate the mycorrhiza (Plate 2, Fig. 4). Mycorrhizas have been observed also in the related species *R. serbica* (Rakic & al. 2009).

Observations of the natural populations have shown that they are dominated by adult plants. Young plants were very few and no seedlings could be found. Demographic studies of the sister species *Ramonda myconi* are in concordance with the present study: populations of this species exist due to the adult, well-adapted plants (Pico & Riba 2002). Young plants of *H. rhodopensis* seldom survive, so vegetative reproduction and preservation of the existing plants is of extreme importance for conservation of the species. In spite of the desiccation tolerance of adult plants, it is not certain that seedlings are desiccation tolerant. Thus drought may be a limiting factor in nature. Another possible explanation is the lack of suitable surfaces, where the seedlings can get established, supposedly as in the case of *R. myconi* (Pico & Riba 2002) – the steep, rocky slopes are often flooded. Taking into account that the species is self compatible, other possible explanation of its low survival rate can be potential inbreeding depression. This could be regarded as a direction for future studies. The low survival rate of the seedlings in culture could be explained by the lack of symbiotic fungi.

Conclusion

Although the populations are not endangered presently and the species is categorized as Least Concern (Petrova & Vladimirov 2009), picking of adult plants is the main hazard factor for the balance in the populations. *Ex situ* collections and *in vitro* cultures are extremely important for investigation, but future studies should also include aut- and sinecological factors, such as pollinating agents, symbiotic or antagonistic relationships, demographic structure of the populations, possible genetic sequences of self-compatibility, and effectiveness of the mechanisms preventing autogamy. The protection status of *Haberlea rhodopensis* should also correspond to the tendencies in the development of natural populations.

Plate I

Plate I. Fig. 1. Typical microhabitat of *Haberlea rhodopensis*; **Fig. 2.** The flowers bear features of bee pollination syndrome; **Figs 3** and **5**. Floral mechanism – weak protandry and strong herkogamy; **Fig. 4**. Plants transferred in spring and early summer, especially if taken with the moss cushion, were doing very well *ex situ;* **Fig. 6**. Pollen tube growth.

Plate II

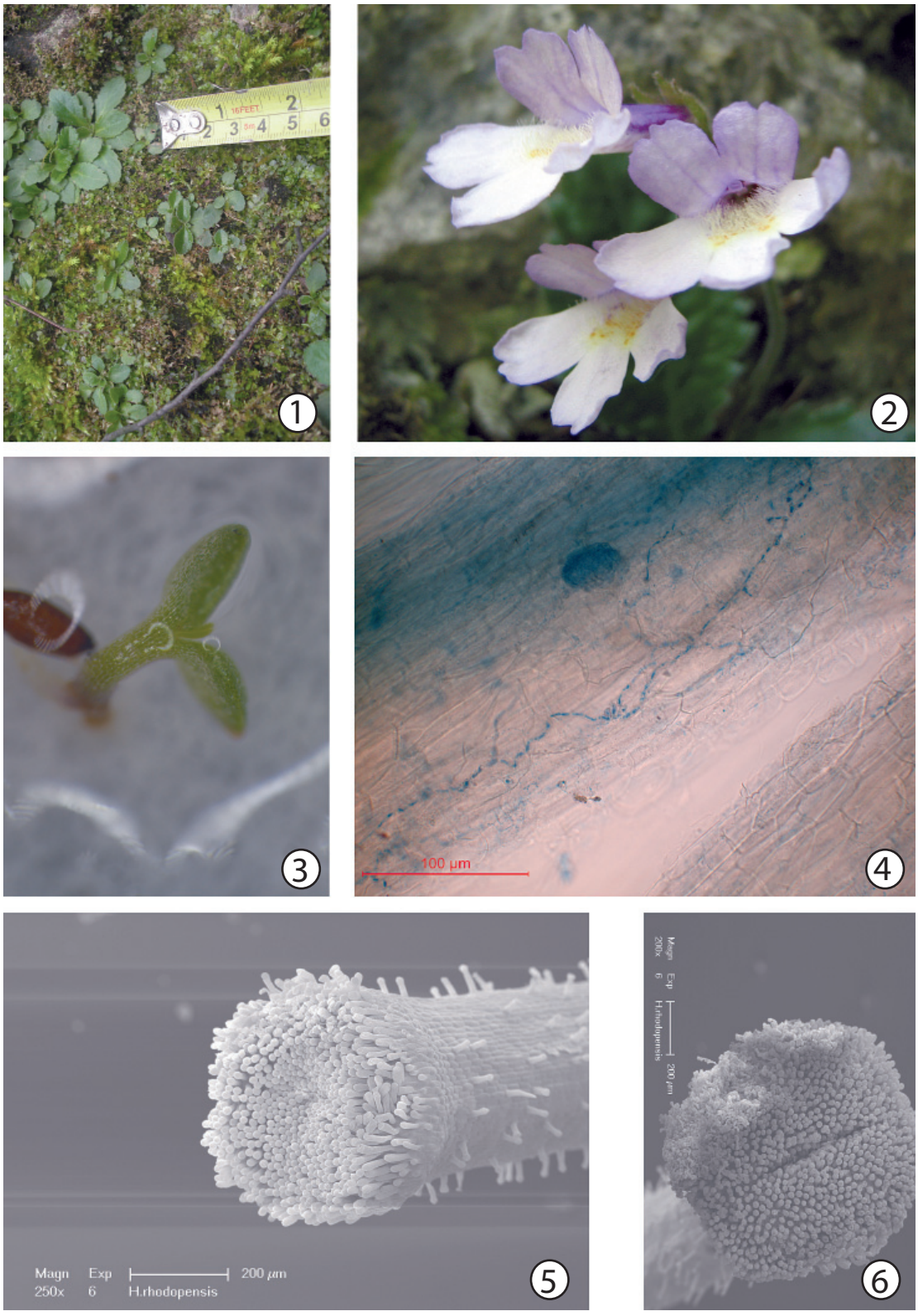

Plate II. **Fig. 1**. *Haberlea rhodopensis* has good ability for vegetative propagation; **Fig. 2**. The flowers bear features of bee pollination syndrome; **Fig. 3**. Seedling of age about four five months; **Fig. 4**. Root microscope preparations clearly show the structures, which indicate the mycorrhiza; **Figs 5** and **6**. Changes in the stigma area indicating receptivity are visible on the microscopic pictures.

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