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Haberlea rhodopensis and it's relatives on the Balkans – what do we know about these peculiar *Gesneriaceae* members of so far?

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Abstract

The so called "Resurrection plants" are extensively studied. On the Balkans beside *Haberlea rhodopensis* occur *Ramonda nathaliae*, *R. serbica* and *Jankaea heldreichii*. The aim of the present paper is to review the current knowledge about the Tertiary relicts of *Gesneriaceae* growing on the Balkans, to analyze comparatively the existing literature data and to outline implications for further research.

Key Words: *Haberlea, Ramonda, Jankaea,* Balkans, review

Introduction

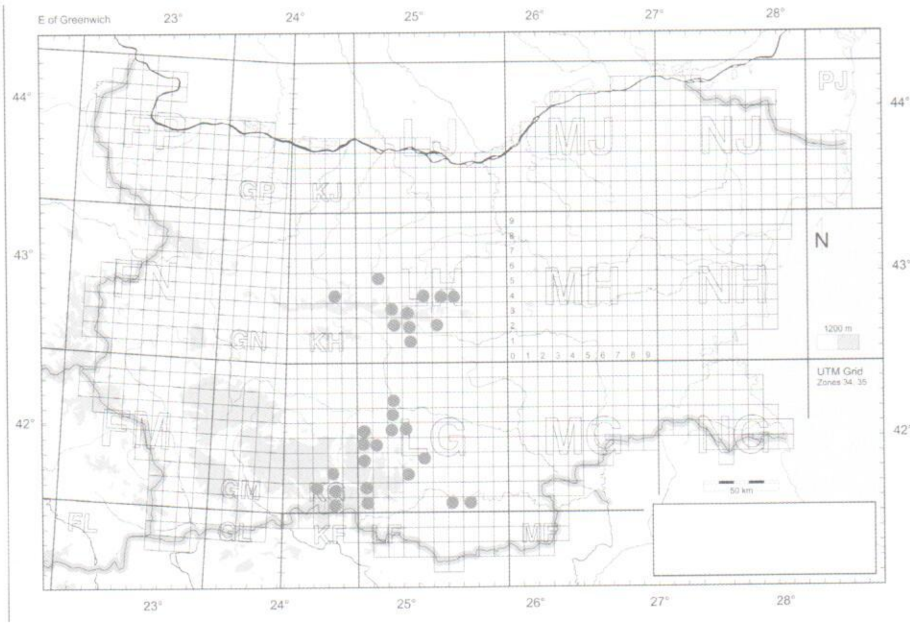
The so called "resurrection plants" are extensively studied. Among them *Haberlea rhodopensis* (Figure 1) gets the focus of the researchers' attention with fundamental and practical approach in different aspects e.g. desiccation tolerance, physiological and biochemical studies, proteoimics and metabolomics, molecular studies, population diversity studies, plant transformation, bioactive compounds and their putative application in pharmacology, veterinary medicine and cosmetics (1). On the Balkans beside *Haberlea rhodopensis* Friv. occur *Ramonda nathaliae* Pančić & Petrovič, *R. serbica* Pancic and *Jankaea heldreichii* (Boiss.) Boiss. (2) and *Ramonda myconi* L. (Rchb.) is distributed at the Iberian peninsula.

Figure 1. *Haberlea rhodopensis* Friv. (*Gesneriaceae*)

The aim of the present paper is to review the current knowledge about the Tertiary relicts of *Gesneriaceae* growing on the Balkans, to analyze comparatively the existing literature data and to outline implications for further research.

Tectonic and climatic changes of the habitats occupied by *Haberlea rhodopensis* and its relatives on the Balkans

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) (Map 1), *Ramonda* (Balkans and Iberian Peninsula) and the monotypical *Jankaea* (in Greece) (3).



Map 1. The distribution of *Haberlea rhodopensis* Friv. (*Gesneriaceae*) in Bulgaria

The Tertiary morphostructures of the contemporary Bulgarian lands is characterized by complicated tectonic conditions and active vertical and horizontal movements.

During the Paleogene begins the formation of the recent blocks of the Rhodopean massif. The Western Rhodopes are already existing as a dry land during the Miocene. At Tortonian – Sarmathian age the Rhodopean massif is under the influence of slow uplifting combined with formation of wide denudational plane at 700 m a.s.l. (4).

According to Kanev (5), in the late Eocene, the morphostructures of West and Central Stara Planina evolve as a stable continental land. During the Miocene the morphological area of the Balkan range between Belogradchik and Varbitsa (Marash Gorge) were sustained dry land.

The neotectonic evolution and forming of the recent geomorphology of Bulgaria begins at the end of the Oligocene. The local geodynamic conditions of submeridional extension at the early Miocene leads to breaking of the crust in the area of abutting Rhodopean and Sredna Gora massifs and the appearance and development of the upper Thracian depression (6).

The climate specifics during the geological period when these *Gesneriaceae* members established and evolved on the Balkans had been an important factor for their evolution. Selected mega- and microfloras from Central and Southern Europe have been analysed for the time interval Tortonian/Pannonian (MN 9–12) with the Coexistence Approach to obtain quantitative palaeoclimate data. The results give a geographically differentiated picture of the late Miocene climate in the circum-alpine area. The obtained data show an overall warm, humid, and homogenous climate with very low latitudinal and longitudinal gradients; there is no indication of a Mediterranean type of climate. Palaeogeographic influences on climate patterns are evident; the Pannonian lake caused mild winters and a lower seasonality of temperature in the Pannonian basin. Also there are data testifying climate changes of warmer/wetter and cooler/drier periods in combination with frequency oscillations of thermophilous elements. There has been cyclic change in peat-forming vegetation related to oscillations of the groundwater level. As a triggering mechanism, wetter/warmer and drier/cooler climate phases related to orbital precession are probable. In addition, sections sampled at high resolution display small scale climate and vegetational variability. As is shown by the analysis ferns were an important component of the peat-forming vegetation, while outside the mire, a wetland vegetation consisting of pioneers and a mixed mesophytic forest with evergreen shrubs existed. At the end of the period herbaceous vegetation has been developed. The increase of *Asteraceae*, combined with a marked decrease in woody taxa, points to an opening of habitats and a decrease in mean annual precipitation (7-13).

Ivanov (13) compared both macro- and microfloristic plant associations from southeastern Europe, palaeogeographically a transitional belt between Tethyan and Paratethyan realms. The Early to Middle Miocene flora is rich and diverse in thermophilous elements, which formed polydominant mesophytic to hygromesophytic forests. Early Miocene climate was warm and humid with mean annual temperatures mainly above 16 C and annual rainfalls over 1000 mm.The middle Miocene was the warmest period of the whole Miocene, with annual temperatures ranging from 17 °C to 19 °C and winter temperatures from 7 °C to 12.5 °C. Climatic changes after the Miocene climatic optimum caused changes in floristic composition and vegetation structure. The vegetation shows a decreasing trend in abundance of palaeotropic and thermophilous elements, reduction of macrothermic elements, and disappearance of evergreen laurel forests. Together with these changes is a corresponding increase in the role of arctotertiary species in plant communities, and they became dominants in mesophytic forests.The available data indicate that major vegetation changes occur in the late Miocene. This period is characterized by more diverse climatic conditions, which were directed by global climatic changes and probably complicated by regional palaeogeographic reorganizations and tectonic processes. Slight cooling and some drying is recorded for the beginning of the late Miocene, followed by fluctuations of palaeoclimate parameters observed which display cycling change of humid/dryer and warmer/cooler conditions. In these ecological conditions the members of *Gesneriaceae* have evolved specific adaptations that appeared to give them the opportunity to survive the harsh conditions of the Quaternary glaciations.

The mild environmental conditions of Tertiary were dramatically changed during Quaternary. Microfossils (palynological research) were used to trace the changes of vegetation and climate during the late glacial and Holocene were traced (the last 14000 years). During the late glacial interstadials thermophylous trees (oak, hazel, lime, hornbeam etc.) were preserved in small groups among herb vegetation at 800 – 1000 m a. s. l. (e. g. in the protected from the harsh conditions humid valleys in the Rhodopes). During the late glacial stadials the herb vegetation readvanced replacing trees. The onset of the Holocene (11 400 years ago) was characterised by a quick amelioration of the climate that favored the distribution of mixed oak forests at lower altitude. Above them were distributed groups of birch and pines. This vegetation pattern lasted for 4 000 years. During the climatic optimum all deciduous and coniferous species increased in the mountains and developed the pattern that we more or less observe nowadays (14-22).

Physiological and biochemical adaptations with emphasis on desiccation tolerance

The lipid and sterol composition of leaves of *Haberlea rhodopensis* and *Ramonda serbica* and their changes at different water deficits and restoration from anabiosis in light and dark were investigated. Lipid and sterol composition changes more drastically at 50 % water deficit, while at 87 % water deficit (air-dried plant) it is similar to that of fresh plants. This is an indication that there is almost full adaptation of the investigated plants in the stage of anabiosis. The restoration in light leads to lipid and sterol composition similar to that of the fresh plants, while restoration in dark leads to a composition similar to that of half-dried plants (23).

The dynamics of key components involved in leaf tissue antioxidant systems under desiccation in the resurrection plant *Haberlea rhodopensis* and the related non-resurrection species *Chirita eberhardtii* are studied. Levels of H_2O_2 decreased significantly in both species, but that accumulation of malondialdehyde was much more pronounced in the desiccation-tolerant *H. rhodopensis.* A putative protective role could be attributed to accumulation of total phenols during the late stages of drying. The total glutathione concentration and GSSG/GSH ratio increased upon complete dehydration of *H. rhodopensis.* Their data on soluble sugars suggest that sugar ratios might be important for plant desiccation tolerance (24).

Georgieva & al. (25) report on the application of a cDNA-AFLP analysis to examine gene expression in leaves of *Haberlea* during dehydration. Twenty transcripts among 33 sequenced cDNA fragments appear to be involved in energy metabolism, transport, cell-wall biogenesis, signal transduction, or are probably transcription regulators according to their putative function. Two up-regulated transcripts *Hrh*DR8 and *Hrh*DR35 encoding putative succinate-dehydrogenase and xyloglucan endotransglucosylase/hydrolase (XTH), respectively, were induced during early stage of dehydration, persist in desiccated state, and subsequent rehydration of *Haberlea.*

Haberlea exposed to drought stress, desiccation, and subsequent rehydration showed no signs of damage or severe oxidative stress compared to untreated control plants. Transcriptome analysis by next-generation sequencing revealed a drought-induced reprogramming, which redirected resources from growth towards cell protection. Repression of photosynthetic and growth-related genes during water deficiency was concomitant with induction of transcription factors presumably acting as master switches of the genetic reprogramming, as well as with an upregulation of genes related to sugar metabolism, signaling, and genes encoding early light-inducible, late embryogenesis abundant and heat shock proteins. At the same time, genes encoding other LEA, HSP, and stress protective proteins were constitutively expressed at high levels even in unstressed controls. Genes normally involved in tolerance to salinity, chilling, and pathogens were also highly induced, suggesting a possible cross-tolerance against a number of abiotic and biotic stress factors. A notable percentage of the genes highly regulated in dehydration and subsequent rehydration were novel, with no sequence homology to genes from other plant genomes. This observation, together with the complex antioxidant system and the constitutive expression of stress protective genes suggests that both constitutive and inducible mechanisms contribute to the extreme desiccation tolerance of *H. rhodopensis* (26).

It has bee shown that diurnal patterns of CO_2 exchange and fluctuations of tissue malic acid concentrations were investigated in *Haberlea rhodopensis* grown under irradiances of 30 or 300 μmol(photon) m^{-2} s^{-1} at transition from biosis to anabiosis and *vice versa.* Different degree of CAM-cycling were exhibited under well-watered conditions and extreme desiccation under both irradiances. The CAM-cycling was proved as efficient mechanism of saving water (27). Thermoluminescence glow curve parameters were used to access the functional features of PS II in *Haberlea rhodopensis.* The authors consider the observed unusual TL characteristics of *Haberlea rhodopensis* reflect some structural modifications in PS II (especially in D1 protein), which could be related to the desiccation tolerance of this plant. This suggestion was supported by the different manner in which dehydration affected the TL properties in desiccation-tolerant *Haberlea* and desiccation-sensitive spinach plants (28).

PSII photochemistry is only affected at leaf relative water contents lower than about 20 %, thus confirming that primary photosynthetic reactions are resistant to drought. Interestingly, the effect of leaf desiccation on photosynthetic capacity, is identical to that observed for three non-resurrection C_3 mesophytes. This demonstrates that the photosynthetic apparatus of *H. rhodopensis* is not more resistant to desiccation when compared to other C_3 plants. Since the leaf area decreases by more than 50 % when the leaf relative water content is reduced to about 40 % during drought it is supposed, that *H. rhodopensis* leaf cells avoid mechanical stress (29).

A new instrument (M-PEA), which measures simultaneously kinetics of prompt fluorescence (PF), delayed fluorescence (DF) and modulated light reflection at 820 nm (MR), was used to screen dark-adapted leaves of the resurrection plant *Haberlea rhodopensis* during their progressive drying, down to 1 % relative water content (RWC), and after their re-watering. This is the first investigation using M-PEA, which employs alternations of actinic light

(627-nm peak, 5000μmol photons $m^{-2}s^{-1}$) and dark intervals, where PF-MR and DF kinetics are respectively recorded, with the added advantages: (a) all kinetics are recorded with high time resolution (starting from 0.01ms), (b) the dark intervals' duration can be as short as 0.1ms, (c) actinic illumination can be interrupted at different times during the PF transient (recorded up to 300s), with the earliest interruption at 0.3ms. Analysis of the simultaneous measurements at different water-content-states of *H. rhodopensis* leaves allowed the comparison and correlation of complementary information on the structure/function of the photosynthetic machinery, which is not destroyed but only inactivated (reversibly) at different degrees; the comparison and correlation helped also to test current interpretations of each signal and advance their understanding. The results suggest that the desiccation tolerance of the photosynthetic machinery in *H. rhodopensis* is mainly based on mechanism(s) that lead to inactivation of photosystem II reaction centres (transformation to heat sinks), triggered already by a small RWC decrease (30).

The functional peculiarities and responses of the photosynthetic system in the *Haberlea rhodopensis* and the non-desiccation-tolerant spinach were compared during desiccation and rehydration. Increasing rate of water loss clearly modifies the kinetic parameters of fluorescence induction, thermoluminescence emission, far-red induced P700 oxidation and oxygen evolution in the leaves of both species. The values of these parameters returned nearly to the control level after 24 h rehydration only of the leaves of HDT plant. PS II was converted in a non-functional state in desiccated spinach in accordance with the changes in membrane permeability, malondialdehyde, proline and H_2O_2 contents. Moreover, the data showed a strong reduction of the total number of PS II centers in *Haberlea* without any changes in the energetics of the charge recombination. It is considerd *Haberlea* to reflect some specific adaptive characteristics of the photosynthetic system. These features of *Haberlea* can explain the fast recovery of its photosynthesis after desiccation, which enable this HDT plant to rapidly take advantage of frequent changes in water availability (31). During the first phase of desiccation the net CO_2 assimilation decline was influenced by stomatal closure. Further lowering of net CO_2 assimilation was caused by both the decrease in stomatal conductance and in the photochemical activity of photosystem II. It could be suggested that unchanged chlorophyll content and amounts of chlorophyll-proteins, reversible modifications in PSII electron transport and enhanced probability for non-radiative energy dissipation as well as increased polyphenolic synthesis during desiccation of *Haberlea* contribute to drought resistance and fast recovery after rehydration (32). The differences in some morphological and physiological characteristics of sun- and shade-adapted *Haberlea rhodopensis* plants were compared. Changes in the photosynthetic activity, electrolyte leakage from leaf tissues, malondialdehyde content (MDA) and leaf anatomy were studied at different degrees of desiccation as well as after rehydration of plants. The MDA content in well-watered sun *Haberlea* plants was higher compared to shade plants suggesting higher lipid peroxidation, which is commonly regarded as an indicator of oxidative stress, but desiccation of plants at high light did not cause additional oxidative damage as judged by the unaffected MDA content. The electrolyte leakage from dried leaves from both shade and sun plants increased fourfold indicating similar membrane damage. However, the recovery after rehydration showed that this damage was reversible. Well-watered sun plants had higher photosynthetic activity probably due to the larger thickness of the mesophyll layer in such plants. On the other hand, desiccation at high light reduced CO_2 assimilation which was in accordance with the stronger reduction of stomatal conductance. Stomata were visible only on the abaxial side of sun leaves having also higher abundance of non-glandular trichomes. Increased trichomes density and epicuticular waxes and filaments upon desiccation could help plants to increase reflection, reduce net radiation income, slow down the rate of water loss and survive adverse conditions (33).

The effect of prolonged light deprivation on ultrastructure, pigment composition and functions of photosynthetic apparatus of *Haberlea rhodopensis* was studied. *Haberlea rhodopensis* demonstrated extraordinary ability to preserve its photosynthetic machinery intact despite complete absence of light. During the first 4 weeks of light deprivation, is observed preservation of pigment content, chloroplast ultrastructure and a decrease in the rate of CO_2 assimilation. The signs of dark-induced senescence were observed only after the fourth week. In comparison with other plants like common bean and *Arabidopsis*, the processes of dark-induced senescence were very slow and the plants still can recover even after 6 months of light deprivation (34).

The ecophysiological responses of *Haberlea rhodopensis* showed that this plant could tolerate water deficit and both leaves and roots had high ability to survive severe desiccation. There was a good correlation between the root respiration and water content. The results showed that the plasticity of adaptation in leaves and roots were different during extreme water conditions. Roots were more sensitive and reacted faster to water stress than leaves, but their activity rapidly recovered due to immediate and efficient utilization of periodic water supply (35).

Haberlea rhodopensis plants, growing under low irradiance in their natural habitat, were desiccated to air-dry state at a similar light intensity under optimal (23/20°C, day/night) or high (38/30°C) temperature. Dehydration of plants at high temperature increased the rate of water loss threefold and had a more detrimental effect than either drought or high temperature alone. Water deficit decreased the photochemical activity of PSII and PSI and the rate of photosynthetic oxygen evolution, and these effects were stronger when desiccation was carried out at 38°C. Some reduction in the amount of the main PSI and PSII proteins was observed especially in severely desiccated *Haberlea* leaves. The results clearly showed that desiccation of the homoiochlorophyllous poikilohydric plant *Haberlea rhodopensis* at high temperature had more damaging effects than desiccation at optimal temperature and in addition recovery was slower. Increased thermal energy dissipation together with higher proline and carotenoid content in the course of desiccation at 38°C compared to desiccation at 23°C probably helped in overcoming the stress (36).

The chlorophyll content in *Ramonda serbica* and *R. nathaliae* intact plants and cut off leaves during dehydration and after rewatering was studied. Rehydration and chlorophyll resynthesis were not light depended. Levulinic acid inhibited completely the chlorophyll resynthesis indicating that the regulatory site on the biosynthetic pathway was located before protoporphyrin formation. Chlorophyllase activity changed during the dehydration and rehydration, but the chlorophyll content was not in direct correlation with this enzyme activity (37).

Plants of *Ramonda serbica* were dehydrated and subsequently were rehydrated. Plasma membranes were isolated from leaves. Dehydration caused a general decrease in the unsaturation level of individual phospholipids and total lipids as well. Upon rehydration the lipid composition of leaf plasma membranes restored very quickly approaching the levels of well-hydrated leaves (38).

Experimental test was conducted with leaves of *R. serbica* collected from nine populations in Kosovo, three in Albania and two in Macedonia, and for *R. nathaliae* four populations in Macedonia. Results revealed that the pigment contents (Total chl + Carot) and the ratio chlorophyll a/b differ in the populations. These studies clearly indicate that the *Ramonda* plants from different ecological habitats there have been changes of photosynthetic pigment contents (39).

The poikilohydric *Gesneriaceae* of the Balkan peninsula are characterized by ahemicryptophytic life form with rosettes of evergreen leaves as well as by shared morphological features of these leaves and their surface structures. The differences in indumentum thickness, stomata distribution, mesophyl compactness, and the number of celltiers in the palisade tissue correspond to a lower or higher degree of xeromorphic adaptation.The varying degree of xeromorphism of these plants is a direct adaptive response to the specific conditions of their respective habitats.The structural differences between these desiccation tolerant "resurrection plants", that we use to regard as a "secondary" set of adaptations, reflect small-scale ecological and chorological divergences: the least xeromorphic, or rather, the most mesomorphic of them, *Haberlea rhodopensis,* inhabits moist, shaded chasmophytic habitats, chiefly in forest, at altitudes of up to 2000 m; the meso-xeromorphic *Ramonda serbica* is frequent on similarly protected rocky ground in thermophilous forests and on north-facing slopes in gorges, but does not exceed an altitude of 1800 m. *R. nathaliae* and *Jancaea heldreichii* are more markedly xerophytic. *R. nathaliae* is characterized by a xeromorphic mesophyll and a dense hair cover on the abaxial leaf face, which enables its survival under environmental stress conditions (water deficit on shallow, rocky soil, both limestone and serpentine, at altitudes up to 2250 m). The xeromorphism of *Jankaea heldreichii* expressed in its small and thickly velvety leaves, enables this stenendemic orophyte to survive under the Mediterranean climate conditions of mt Olympus (40).

The resurrection flowering plant *Ramonda serbica* inhabits the shallow organo-mineral soil that develops in crevices on northern-facing carbonate rocks in the gorges in the Balkan Peninsula (41). This type of soil represents a complex substrate whose physical and chemical properties were found to be well suited to the most important requirements for the growth and development of *R. serbica* as well as for the plant's survival in the state of anhydrobiosis in periods of drought stress. Considerable amount of organic matter in the soil resulted in the high field capacity as well as the slow changes in the amount of its available water. The suitable soil hydric status, based on the organic remains, supports the slow dehydration of this poikilohydric plant, which is extremely important in allowing the activation of the plant's protective mechanisms. The pH of the soil solution was slighty alkaline (7.7) mostly due to carbonates in its crystallographic structure. The adequate content of nutrients in the leaves points to efficient mineral consumption by the plant roots. The sufficient bioavailability of nutrients and water was also improved by vesicular-arbuscular mycorrhiza detected in *R. serbica* roots. Ecological factors contributing to fecundity showed a high degree of between-year variability by *R. myconi* (42). Pre-dispersal fruit predation had a minor influence on total reproductive output, and most of the variability was found among individuals within populations and years. Spatio-temporal variability in growth and survival was rather low but significant, whereas recruitment showed important between-population variability. Among-year variability in fecundity and growth was related to climatic fluctuations on a regional scale, notably rainfall and temperature in a particular period, while the spatial variability in survival and recruitment was explained by within-population (patch) habitat quality. Although *R. myconi* is able to withstand repeated periods of drought, water availability seems to be the most important factor affecting plant performance in all the study populations. These findings suggest that the long-term persistence of species showing remnant population dynamics in habitats under the influence of Mediterranean climate might be threatened by increased aridity as a result of climate change.

The main life-history features of *Ramonda myconi* are the great longevity of adult plants and the high mortality of seedlings (43). The exact life span of *Ramonda myconi* is not known but some plants in the Royal Botanic Garden of Edinburgh (Edinburgh, UK) have at least more than 30 years (M. Möller & Q. Cronk, personal communication). Production of 2–3 new leaves may occur every year. Growth rates were generally low among seedlings and juveniles. Seedlings only established as juveniles and the establishment of seedlings as small adult plants was not observed in any of the study years. Juveniles only showed growth to small adult plants and not to greater adult sizes. Adult plants did not show dramatic changes in plant size and basically tended to remain in the same stage. This was particularly true for the adults of the largest size class which showed stasis values as high as 90 %. Stasis of small and medium adults was lower than that of large adults. The proportion of small and medium adults that increased or decreased in size was as high as 50 %. Fecundity increased with plant size although the mean number of seedlings per plant and per size class was low. Overall, the population growth rate ranged from a low of 0.79 to a high of 1.06. local *R. myconi* populations tend to decline over time but with a long time to extinction, so its persistence is determined by the persistence of its remnant local populations.

Sucrose accumulation is connected to desiccation tolerance in *Gesneriaceae*; the presence of raffinose may be a pre-adaptation since this sugar prevents crystallization of sucrose during drying. In leaves of *Ramonda nathaliae* subjected to various desiccation regimes sucrose was the predominant soluble carbohydrate and its level steadily increased during desiccation. Starch amounted to ca 2 % in control leaves and disappeared completely within 8 days of desiccation. Considerable amounts of raffinose and smaller amounts of its precursor galactinol showed only minor changes upon desiccation. Similar results were obtained when excised leaves of *Ramonda nathaliae, Ramonda myconi* and *Haberlea rhodopensis* were subjected to desiccation (44).

Ramonda serbica was subjected to dehydration and then rehydrated by rewatering. The decrease in the osmotic potential at full turgor indicates that an osmotic adjustment came into play. The osmotic adjustment in the dried leaves was due primarily to the high concentration of inorganic ions (71 % of total solutes), especially K+and Cl- other detected compounds such as soluble sugars and free amino acids gave a rather low contribution to the osmotic potential at full turgor of the desiccated leaves. Generally, upon rehydration all the osmotically active substances almost returned to their initial concentrations. The presence of increased amounts of sucrose detected during desiccation is discussed in relation to its role in membrane stabilisation (45).

Comparative analysis of the electrolyte efflux, as a screening test of the membrane tolerance to water stress, was carried out in poikilohydric plants *Ramonda serbica* and *Ramonda nathaliae* and homoiohydric plant *Saintpaulia ionantha* Wendl from the same family (46). The high degree of solute leakage in the East-African drought-intolerant *Saintpaulia ionantha* points to the loss of membrane integrity. In contrast, Balkan endemites *Ramonda serbica* and *R. nathaliae* show high resistance to water stress due to the specific constitutional drought tolerance mechanisms.

Most studies are dedicated to drought tolerance mechanisms. There is some chorological data (47, 48). Scarce is the data on the autecological specifics e.g. microhabitat requirements and tolerance, exposition, plant communities, population dynamics, lifespan and ontogenesis peculiarities and further research efforts should be towards these problems.

Reproductive co-adaptations and co-evolution

The co-evolution with the pollinating agent is a key factor for the adaptive radiation of the plant taxa (49, 50). The functional morphology of the flowers of *Haberlea, Ramonda* and *Jankaea* suggest co-adaptation to *Apoidea* according to the syndromes of pollination described by Faegri & van der Pijl (51) and Proctor & al. (52). The *Apoidea* s.str. diverged from the *Sphecoidea* s.str. in late Secondary and their diversification took place during Tertiary and Quaternary (53-55). The contemporary species can generally be considered as the result of a late Quaternary specification process (consequence of the Würm glaciation). During glaciations following a now relatively well known process, a large ice sheet covered the southern Palaearctic. This northern ice sheet was followed by a large zone of tundra-like ecosystems which is totally unsuitable for survival of numerous species, in particular for the *Apoidea.* The two main consequences of these eco-climatic transformations are the extinction of most stenotypic taxa and the restriction of the more eurytopic species to small disconnected refugia distributed in meridional parts of conti the West-Palaearctic area. Other consequences of glaciations are notably the possibility of slight southward expansion due to the reduction of the sea level. The transformation of the distributions under the pressure of glaciations appears to be clearly favourable to diversification (56).

Studies on pollinators revealed that wild bees were extremely rare and honeybees preferred other plants for foraging than *H. rhodopensis* in the studied communities. *Haberlea rhodopensis* practically received no visits, except for a single nectar-collecting *Bombus hortorum* queen (57).

Jankaea heldreichii, is an insect pollinated plant, but no special relationship between it and its insect partners has evolved (58). Pollinator visits were scarce by bumblebee queens. Anthesis has a prolonged duration up to 10 days and nectar is absent. There is a significant variation in number of seeds in big, medium sized and small capsules. Bagging experiments show absence of spontaneous autogamy.

Flowers of *Ramonda myconii* are hermaphroditic, self-compatible but not capable of autopollination, and mainly visited by bumblebees and syrphids (Riba & Picó, unpublished result).

It is obviously necessary to focus more research efforts on the reproductive biology as the data so far is scarce and insufficient for elucidation of the enigma.

Karyosystematic and DNA studies

Key studies from evolutionary point of view are the chromosome numbers of taxa and they are listed by Moore (59) as follows:

Haberlea rhodopensis Friv. 38 Bu Borhidi, 1968; 48 Gr, Contandriopoulos1967

Ramonda myconi (L.) Reichenb. 48 Ga, Contandriopoulos 1967

R. nathaliae Pancic § Petrovic 48 Ju, Gr, Contandriopoulos 1967

R. serbica Pancic c. 96 Gr, Contandriopoulos 1967

Jankaea heldreichii (Boiss.) Boiss. 56 Gr, Contandriopoulos 1967

Several ploid levels are established for *Haberlea rhodopensis* and it is curious fact that the level of 2n=48 is reported both for Bulgaria and Greece (60). In Bulgaria is reported also the level of 2n=38 Moore (59). Possibly this fact of two ploid levels correlates with the established morphological differences described as separate subspecies: var. *rhodopensis* and var. *ferdinandi-coburgii* (Flora Republicae Bulgaricae 1995). Polysomaty occurred in all root-tips of *Haberlea rhodopensis* examined by Milne (61): a majority of figures had 2n=44 but others showed numbers varying from 2n=c.30 to c.50

The genus *Ramonda,* including three preglacial paleoendemic species on the Balkan (*Ramonda nathaliae* and *Ramonda serbica*) and Iberian peninsulas (*Ramonda myconi*) also shows different ploidy levels (62). *R. nathaliae* and *R. myconi* are diploid species ($2n=2x=48$) while *R. serbica* is hexaploid ($2n=6x=144$). In one population of *R. serbica* the DNA content ranged from 2C=7.65 to 11.82pg, revealing different ploidy levels among its individuals. In sympatric populations genome size was intermediary between the diploid and hexaploid classes which indicates the hybridization ability between *R. serbica* and *R. nathaliae.* It appears that polyploidization is the major evolutionary mechanism in the genus *Ramonda.*

Cytogenetical study of natural populations showed polyploid forms as a mixture (2n=72) and (2n=96) with a predominance of the most frequent form (2n=96) in *Ramonda serbica* (63).

Further on the initial study using molecular methods included three geographically isolated populations from Rhodopi and Stara Planina mountains. It is assesed at DNA level the polymorphism between these populations by internal transcribed spacer 1 (ITS1) sequences. The obtained results demonstrated that the ITS1 regions is suitable as molecular marker for studying genetic polymorphism between *H. rhodopensis* populations but it should be combined with other marker genes that are represented in a single copy in the plant genome. The initial results indicate that it could be expected unusual patterns of polymorphisms distribution between *Haberlea rhodopensis* populations (63). RAPD markers provided a useful technique to study genetic diversity in *Ramonda serbica* and *R. nathaliae* populations. This technology allows the identification of different populations as well as the assessment of the genetic similarity among different populations (63).

Dubreuil & al. (64) used RAPD and chloroplast markers to assess the patterns of genetic structure in eight mountain populations of *Ramonda myconi* covering almost the full species range, to identify the main historical processes that have shaped its current distribution and to infer the number and location of putative glacial refugia. While no cpDNA variation was detected, the species had relatively high levels of RAPD variation. Maximum levels of diversity were found within populations (71 %), but there was also a significant differentiation between geographical regions (20 %) and among populations within regions (9 %). A spatial AMOVA identified three main groups of populations, corresponding to previously recognized centers of endemism and species richness. In addition, was found a marked geographical pattern of decreasing genetic diversity and increasing population differentiation from west to east. The results support a complex phylogeographic scenario in the Iberian peninsula of "refugia-within-refugia" and suggest that the higher diversity observed in western regions might be associated with prolonged and more stable climatic conditions in this area during the Quaternary.

A large number of genes have been identified which may play a role in underpinning the ability of the plant tissues to survive severe tissue water loss. There have been a number of elegant experiments which have used tissue culture to study metabolism in resurrection plants. The time is now ripe for the large scale molecular analysis of the poikilohydric ability of these plants using tissue culture of *Craterostigma plantagineum*, *Haberlea rhodopensis* and *Ramonda myconi* (which have been studied as model resurrection plants) as a major tool to drive research forward. Large-scale isolation of drought stress associated genes with unknown biological roles requires functional analysis. It is shown that conventional genetic transformation techniques, via *in vitro* plant regeneration systems, still represent an unavoidable part of the high-throughput functional genetics analyses. Similarities and differences in tissue culturing and genetic transformation of these resurrection plants, as a consequence of their common physiological specialisation, are summarized, and future prospects of advances in this area of research are discussed (65).

Secondary compounds, their biological activity and application

The presence of six multiple superoxide dismutase isoforms in the protein extract from fresh leaves of *H. rhodopensis* is revealed, four of them belonged to CuZn-SOD isoforms, one belonged to Mn-SOD and one - to Fe-SOD. The same method showed one form of nonspecific guaiacol peroxidase and two multiple isoforms of ascorbate peroxidase (66).

The antioxidant effect total extract of *Haberlea rhodopensis* was assessed by determination of total (Cu-Zn and Mn) superoxide dismutase (SOD) activity The results of experiment show higher SOD-like activity for 1 ml extract compared to a referent compound TroloxTM (water- soluble vitamin E analog). This facts can be explained with probable existence of the some phytochemicals as flavonoides and antocianines (cianidine and. quercetine) into the total extract of *H. rhodopensis* which are known as strong scavenging and antioxidant agents (67).

Phytochemical profiling of a MeOH extract from *Haberlea rhodopensis* afforded three new flavone *C*-glycosides, hispidulin-8-*C*-(2″-*O*-syringoyl)-*β*-glucopyranoside, hispidulin 8-*C*-(6-*O*-acetyl-*β*-glucopyranoside), and hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*β*-glucopyranoside), along with two known phenolic glycosides, myconoside and paucifloside (68).

GC–MS metabolic profiling of the apolar and polar fractions from methanolic extracts of *Haberlea rhodopensis* revealed more than one hundred compounds (amino acids, fatty acids, phenolic acids, sterols, glycerides, saccharides, etc.). Bioactivity assays showed that the polar fractions possessed strong free radical scavenging activity, while both the polar and apolar fractions failed to provoke any significant cytotoxic effects against the tested cell lines. Five compounds possessing antiradical activity were identified – syringic, vanillic, caffeic, dihydrocaffeic and p-coumaric acids (69).

High-performance liquid chromatography analysis was performed to quantify the major phenolic acids and flavonoids. The results suggested the possibility of practical application of *H. rhodopensis* leaf extracts because of the established free radical-scavenging activity (70).

The effect of *Haberlea rhodopensis* and vitamin C on the frequency of chromosome aberrations in rabbit's peripheral blood lymphocytes after *in vitro* gamma irradiation was compared. Results demonstrated that *H. rhodopensis* extract reduced the frequency of chromosome aberrations, especially double chromosome fragments and dicentrics, as well as aberrant cells *and* was found to be more effective in reducing aberrant cells than vitamin C. The effect of *H. rhodopensis* and vitamin C on the frequency of dicentrics and double acentric fragments was similar. It can be concluded that the extract of *H. rhodopensis* showed a radioprotective potential (71). Anticlastogenic and radioprotective effects of the total extract of *Haberlea rhodopensis* were investigated using chromosome aberration assay on lymphocytes after irradiating peripheral blood samples at gamma radiations from rabbits pretreated with different doses of the total extract by the intramuscular route. The gamma radiations induced the occurrence of dicentric chromosomal aberrations in lymphocytes in a dose-dependent way but the HR pretreatment of rabbits 2 hours before has greatly alleviated the frequency of dicentric cells whatever the extract doses used. However, the decrease in the dicentric anomalies was proportional to the injected HR doses, the maximal radioprotective effect being achieved with the highest HR dose. Furthermore, the HR pretreatment has also reduced the radiation-induced oxidative stress by significantly preserving the antioxidant SOD and CAT activities and significantly reducing the MDA formation. These results demonstrate the highly radioprotective and antioxidant potential of the *Haberlea rhodopensis* in rabbits but the identification of the protective compounds need further investigations (72). New Zealand white rabbits were intramuscular administered with total extract of HR then treated with cyclophosphamide. Extract of *Haberlea rhodopensis* inhibited the frequency of chromosomal aberrations induced by Cyclophosphamide. The results showed that CP at a dose of 50 mg/kg, i.m. signifycantly inhibited the activities of SOD and increased MDA contents in rabbits blood. On the other hand, HR antagonized the reduction of the activities of SOD and GPx, and the increase in MDA contents. In conclusion, the results of this in vivo study show that HR has antimutagenic potential against carcinogen CP (72). Also the protective ability of HRE against oxidative damage induced by a non-lethal dose of 60Co-γ-rays was evaluated. Results show that administration of HRE before and after irradiation decreased the MDA level and increased SOD and CAT activity, thus providing protection against the radiation-induced decrease in antioxidative ability and increase in lipid peroxidation.This finding supports the idea that HRE is a potent free radical scavenger and antioxidant (73).

A total extract from *Haberlea rhodopensis* leaves was tested for antibacterial activity on some standard and wild pathogenic bacterial strains. The results show that the inhibition of the bacterial growth was more pronounced on *Staphylococcus aureus* than on Gram–negative strains – *Pseudomonas aeruginosa* and *Escherichia coli* (74).

Haberlea rhodopensis extract is rich of a caffeoyl phenylethanoid glycoside (myconoside). Peroxide-stressed normal human dermal fibroblasts treated with the *Haberlea* extract, showed increased collagen and elastin mRNA synthesis. This effect was superior to those obtained with benchmarks retinoic acid and retinol. *Haberlea* extract protected against UV-induced dermis oxidation by 100 %. Finally, the extract was tested in human in a cream against a placebo. The extract can be suggested for anti-ageing treatments, intended for claims such as protection from oxidation, increased skin elasticity and enhanced skin radiance (75).

Haberlea rhodopensis was studied as a potential source of novel cancer modulating drugs. Human embryonic (HEK293 $p53^{+/+}$) and prostate cancer cell lines – LNCaP ($p53^{+/+}$) and PC3 ($p53^{-/-}$) were used as a model to follow the reaction to oxidative and genotoxic stress after pre-treatment with *H. rhodopensis* extract. Oxidative stress was estimated by flow cytometry (FCS) and fluorescent plate reader (FPR) using reactive oxygen species fluorescent dye (H_2DCFDA). UV induced DNA damage was assessed by FCS PUMA (p53 upregulated modulator of apoptosis) expression. Inflammatory pathways were challenged using synthetic peptidoglycan by FCS Act1 expression and NFκB reporter stable cell line. Pre-treated-cells were assessed using DCF dye (FPR and FCS) for ROS (Reactive Oxygen Species) generation after dose-dependent H_2O_2 stress and decreased signal compared to non-treated cells. *H. rhodopensis* had cumulative effect on cell death in PC3 cells. UV-induced genotoxic stress resulted in FCS-detected PUMA upregulation only in PC3. *Haberlea* treated cells had better vitality and their challenge using a bacterial peptidoglycan resulted in an upregulation of Act1, but only in LNCaP cells. The NFκB reporter vector revealed transcription factor activation upon treatment with only peptidoglycan, while *Haberlea* pre-treatment resulted in negative modulation of the NFκB induction. The data show that *H. rhodopensis* extracts have anti-oxidative effect in cancer vs. normal cell lines and differentially modulate distinct cell lines in genotoxic and inflammatory stress, favoring NFκB activation in $p53^{+/+}$ cells, while suppressing its signaling in $p53^{-/-}$ cells (76).

Mother Tincture made from the flowers of *Haberlea rhodopensis* is used lately in Homeopathy (Rumenin, personal communications). *Ramonda myconi* is endemic in the Pyrenees and north-eastern Iberian Peninsula. It has been used as an expectorant and cough remedy, and it is worthwhile considering its indications from a homeopathic point of view, through a proving. To do this, we have followed the criteria of the ECH (European Committee of Homeopathy) described in the Homeopathic Drug Proving Guidelines. From the Mother Tincture, different dilutions CH (7, 9, 15 and 30) have been developed and experienced by ten volunteers, who have monitored the appearance of symptoms. The results indicate that *R. myconi* is especially useful in people who have colds and persistent dry cough, nasal obstruction, headache and urinary symptoms. As regards the skin, the proving has shown eczema and itching and the appearance of itchy scabs on the scalp and genital area. As concomitant symptoms, local or generalized hot flushes, tingling and burning in certain locations, dry mouth and lips without thirst have been described, as well as states of profound drowsiness and apathy. The mental symptoms include anxiety on waking up in the morning, as if something unpleasant is going to happen, dwelling on sad facts of the past, and forgetfulness or avoidance of the remaining tasks. The main reactional modalities are the onset or worsening of symptoms in the morning on waking and in the evening. The symptoms expressed by the volunteers place the remedy within the psoric miasma: reactional modalities, mental and particular symptoms and the polarity of action are within the range of psora. This remedy may be useful in clinical practice and therefore further research would be of interest (77).

Strategies for conservation and ex situ and in vitro propagation

Ramonda serbica is assessed as EN (Endangered) and listed in the Red data book (78, 79). Although *Haberlea rhodopensis* is not included in the new edition of the Red data book (79) due to the fact that it was assessed as LC (Least concerned) (80) it is still rare enough plant that needs protection. It is potentially in hazard being recognized as medicinal plant (81, 82).

An ex situ collection of *Haberlea rhodopensis* from the 12 main localities where the species could be found in Bulgaria is established. A successful, simple and uniform protocol for in vitro propagation for plants from all localities has been developed. Thus, the authors 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 (80).

The most frequently used approach for investigations on gene functions in plant systems is genetic transformation. In this respect, the establishment of *in vitro* systems for regeneration and micro propagation is necessary. On the other hand, *in vitro* cultures of such rare plants such as *Haberlea rhodopensis* could preserve their natural populations. Therefore plant material was collected from one of the natural habitats of the plant. Various plant organs as explants for establishing of aseptic cultures were used. There were great problems with obtaining such cultures due to the morphology of *Habelrea* leaves and roots. Finally, the research team were able to develop in vitro cultures using seeds. The seeds were surface sterilized. The germination was extremely prolonged and of low frequency but after 3 months were obtained several plantlets. The primary plantlets were with extremely reduced size of the leaves, with significant amount of vitrified cultures and low rooting ability on MS and B5 mediums, respectively. On the other hand, on WPM almost all plantlets grew and rooted well (up to 95 %). In addition to the traditional way of multiplication by cuttings were detached the well-developed mature leaves from the rosette plants cut in pieces. First initiation of shoots was visible within a month both at wounded edges and around leaf veins No callus formation during the whole process was observed. The further development of the shoots was relatively faster then that of the roots After subculturing and dividing of the plant clusters it was possible to obtain well-developed plantlets In 1– 1.5 months these plants were fully developed and ready to be transferred to non-sterile conditions The whole procedure takes about 6–7 months. After acclimation, the plants grow normally under greenhouse conditions and outdoors. No regeneration was achieved when MS or B5 were used (83).

The first procedures have been established for *in vitro* micropropagation, plant regeneration, and protoplast transformation of the desiccation-tolerant resurrection plant, *Ramonda serbica*. An integrated optimization of salt and growth regulator composition, and different culture temperatures resulted in an effective tissue culture system, which also prevented the undesirable hyperhydricity and tissue necrosis typical of *Ramonda* tissue cultures. *De novo* regenerated plants developed at high frequencies, which is a precondition for gene transfer and offer a good opportunity to optimize genetic transformation of *R. serbica.* Using leaf blades of *in vitro* propagated plants as donor tissue for protoplast isolation and GFP reporter gene as visually selectable marker gene, PEG-mediated protoplast transfection was also performed. This provides a new tool for studying cellular-level stress responses like stress associated membrane degradation, deficiencies in membrane transport processes (patch clamp technique), or cell cycling abnormalities (84). Establishment of a live collection of in vitro *Ramonda serbica* was started parallel by Dontcheva and co-authors (85). This is accomplished through an in vitro system for regeneration and propagation, modified by the research group. The live collection of in vitro *Ramonda serbica* plants will be a donor for conservation and reintroduction of adapted in vitro plants in their natural endangered habitats and also for physiologicalstudies of drough tolerance, and multidisciplinary comparative analyses (85). A research group at the University of Plovdiv has established a national *in vitro* gene bank for *Haberlea rhodopensis* (25 localities) and *Ramonda serbica* (2 localities) from Bulgaria. The national gene bank is based on original and modified in vitro technologies and can serve as a conservation and biodiversity investigation center for the family *Gesneriaceae.* Basing on the work with *Haberlea rhodopensis* is developed a strategy for conservation and investigation of rare and relic plant species (mapping and exploration of habitats – assessing the local risk of extinction - introducing in an *in vitro* gene bank - model plants for research – adaptation and possible re-introduction in endangered habitats). This strategy can be adapted and used for conservation and investigation of other rare, protected, relic and endemic plants from other regions of Europe and worldwide (86). Additionally a joint research groups have established National in vitro collections of *R. serbica* in Albania and Bulgaria and *R. nathaliae* in Macedonia. In both collections, seeds were used as a convenient starting point for micropropagation in the nutrient medium JG-B. In the Bulgarian in vitro collection, a dry sterilization of seeds was applied for a first time for *Gesneriaceae* family. The micropropagation and conservation of *Ramonda* seeds and plantlets were similar in Albanian and Bulgarian collections. The plantlets in vitro as an explant material were developed in JG-B medium with different phytohormones. The direct organogenesis of two *Ramonda* species is very similar models. The method of conservation in vitro with minimal growth method (modification of nutrient medium) was used (87-89).

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