Micropropagation and in vitro conservation of the rare and threatened plants *Ramonda serbica* and *Ramonda nathaliae*

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Abstract *Ramonda serbica* and *Ramonda nathaliae* are rare and endemo relict plant species from Balkan Peninsula. An efficient micro propagation and in vitro conservation method via direct and indirect organogenesis from seed and leaf explants, respectively, was established in this study. The seed of both *Ramonda* species were collected from different populations in Kosovo, and were germinated in nutrient media JG-B without any phytohormone. The highest number of shoots and multiplication rate was observed on JG-B medium supplemented with BAP and IAA (0.5 mg l^{-1} each), whereas the highest number of leaves per plantlets was found on WPM and RA medium supplemented with BAP and IAA (0.1 mg l^{-1} each). During this stage of micro propagation some significant differences were observed in plantlets from different populations. The indirect organogenesis from parts of leaves of natural plants was not successful due to unavailability of established protocol for disinfections of the plant material. On other hand, parts of leaves from micro propagated plantlets, cultured on MS medium supplemented with different ratio of BAP and NAA, resulted in the highest efficiency for shoot regeneration. In vitro conservation of micro propagated plants at the lower temperature (4°C) had a significantly positive effect for storage of more than 12 months.

Keywords In vitro conservation · Micropropagation · Organogenesis · *Ramonda serbica* · *R. nathaliae*

Abbreviations

JG-B	Jungnickel/Gliemeroth Basal Medium
WPM	Wood Plant Medium
RA	Ramonda medium
MS	Murash and Skoog
BAP	6-benzylaminopurine
IAA	Indole-3-acetic acid
NAA	1-naphtaleneacetic acid

Introduction

Ramonda serbica and *Ramonda nathaliae* are the only representatives of the tropical family *Gesneriaceae* in Kosovo. In Europe this family is represented by three genera (*Ramonda* L.C.M. Richard, *Haberlea* Friv. and *Jankaia* Boiss.) and five species (*Ramonda serbica*, *Ramonda nathaliae*, *Ramonda myconi*, *Haberlea rhodopensis* and *Jankaia heldreichii*). Four of these species occur in Balkan Peninsula, and the only representative in Iberian Peninsula is *R. myconi* (Tutin et al. 1972; Stevanovic et al. 1991). *R. serbica* and *R. nathaliae* are endemic and relict species of the Balkan Peninsula. These plant species are included in the Red Book of Vascular Plants of the Republic of Kosovo (Millaku et al. 2013), whereas only *R. serbica* is listed in the European Red List of Vascular Plants (Bilz et al. 2011).

In recent study, the resurrection plants from *Ramonda* genus have become important models for studying drought tolerance (Quartacci et al. 2002; Veljovic-Jovanovic et al. 2006; Gashi et al. 2013a), photosynthetic abilities during transition from anabiosis (Augusti et al., 2001; Degl'Innocenti et al. 2008; Gashi et al., 2013a), antioxidative

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capacity (Sgherri et al. 2004; Jovanovic et al. 2011) and other specialized and multidisciplinary studies. Other authors have reported a genetic (polyploidy) and morphologic diversity within and between plants of genus *Ramonda* populations (Siljak-Yakovlev et al. 2008; Daskalova et al. 2012; Gashi et al. 2013b; Gashi 2013).

The extreme drought tolerance and the geographic isolation make these species the excellent candidates for isolation of genes involved in the mechanism of drought resistance. In future, the selected genes can be used for production of modified crops to improve crop resistance to stress (Minkov 2009). The elaboration of the tissue culture and the genetic transformation system transmitted by *Agrobacterium tumefaciens* of these plants has opened the opportunity for the functional analysis of genes isolated from desiccation tolerant plants (Tóth et al. 2006, 2009; Toldi et al. 2009, 2010).

The European Gesneriad species are Tertiary relicts (Meyer 1955, 1970; Tutin et al. 1972) and are unique plants that have potential as potted ornamental plants (Byrne 2007; Ferns 1979). Many tropical species of decorative plants of the family *Gesneriaceae* can be propagated using the methods of tissue culture (Tóth et al. 2004; George 1996; Jungnickel and Zaid 1992).

In vitro propagation techniques have been used as an alternative way to propagate and conserve a large number of rare (Bramwell 1990; Holobiuc et al. 2009) and threatened (AmoMarco and Lledo 1996; Dhar et al. 2000; Pence 2005; Rajasekharan et al. 2009) plant species with poor and uncertain responses to conventional methods of propagation (Sarasan et al. 2006). According to Engelmann (1997, 1998) standard culture environment can be effectively utilized for short to medium term in vitro conservation of plant germplasm, through increasing intervals between subcultures in slow growing species.

According to Red Book of Albania (Vangjeli et al. 1993) *Ramonda serbica* belongs to a rare, Balkanic-endemic species, which needs to be protected and preserved as a decorative plant by cultivation. In natural conditions the propagation of *R. serbica* and *R. nathaliae* species from seeds is very slow and difficult (Gashi 2013). Other methods would be welcome to the commercial growers. The in vitro propagated *Ramonda* plants can be used as a donor for plant production in order to conserve and reintroduce the acclimatized plants in their natural endangered habitats and also for physiological studies of drought tolerance.

The aim of this study is to compare the different nutrient media, phytohormones, explants and populations for micro propagation and in vitro conservation of *R. serbica* and *R. nathaliae* plants, and to determine the optimal method for the production of a greater number of plants. This protocol will serve in the future as a base to establish an in vitro genetic bank for germ plasm preservation, to improve the decorative value of these species and to isolate the genes responsible for drought resistance.

Materials and methods

Plant material (seeds collection)

The seeds of *R. serbica* and *R. nathaliae* were collected during July (2012 and 2013) from natural populations of Kosovo. The seeds were collected together with seed capsules in order to be better conserved, and then they were stored at dry and airy place at temperature near 25 °C. Seeds of *R. serbica* were collected from four populations in Sharri Mountains and 5 populations in Albanian Alps, while for *R. nathaliae* seeds were collected from two populations in Shari Mountains (Table 1).

Germination tests

After collection, fine maroon seeds (about 1 mm diameter), which were taken out after the opening of the capsules, were passed through a sieve. Seed viability in conditions in vivo (Petri dishes with double filter paper at temperature 23 °C) was determined, when two green leaves were emerged. For seeds from the populations that had low germination percentage, pre-treatment with 0.3 % potassium nitrate (KNO₃) for 24 h and with 500 ppm gibberellic acid (GA₃) for 24 h was given to increase the germination (Gashi et al. 2012).

Micropropagation method

Direct organogenesis Seeds were used as a convenient starting point for experimental tissue culture because after the germination on the nutrient medium, they can produce contaminant-free plantlets, which may be used as explant material. Before the establishment of in vitro culture, the seeds without preliminary treatment were disinfected using 70 % ethanol for 3 min, followed by 3 washes with sterile distilled water. JG-B (Jungnickel/Gliemeroth Basal) medium (Jungnickel, 1988), pH 4.4–4.6 was used to inoculate the seeds of two *Ramonda* plants.

Stage 1 (Seed germination)

Seed germination in solid JG-B medium without phytohormones. Initially the seeds were inoculated in two 100 ml flasks with respective media. Later the seeds were transferred to test-tubes (3 seeds in each test tube). This procedure facilitates uniform distribution of fine seeds of *Ramonda* plants. The test-tubes were maintained for 6 weeks in a continuous light regime (white fluorescent light of intensity 43.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (Stefanovic and Neskovic 1977; Gashi 2013).

Stage 2 (Seedling development)

Plantlets from stage 1 were transferred in three different nutrient media: **a)** JG-B (Jungnickel/Gliemeroth Basal) medium (Jungnickel 1988) with

Table 1 Geographic origin of *R. serbica* and *R. nathaliae* populations in Kosovo

Code of populations	Species	Populations	Directions	Altitude (m)	UTM-zone 34 (WGS84)
1	<i>R. serbica</i>	Gorge of Zhlebi	N and NE	1,250	X=0440095 Y=4735341
2	<i>R. serbica</i>	Radac	N	545	X=0443216 Y=4731643
3	<i>R. serbica</i>	Gorge river of Sushica	N	676	X=0441054 Y=4727481
4	<i>R. serbica</i>	Canyon of Rugova	N	800	X=0434691 Y=4723960
5	<i>R. serbica</i>	Gorge of Koprivnik	N	750	X=0439892 Y=4719967
6	<i>R. serbica</i>	Gorge of river Prizren	N	530	X=0482119 Y=4671129
7	<i>R. serbica</i>	Gorge of Rusenica	N	1,340	X=0489481 Y=4679712
8	<i>R. serbica</i>	Gorge of Matosi	N	910	X=0492515 Y=4680316
9	<i>R. serbica</i>	Shkëmbi i përgjakur	N	1,170	X=0494025 Y=4679758
10	<i>R. nathaliae</i>	Gllouçicë	N and NE	982	X=0515118 Y=4669685
11	<i>R. nathaliae</i>	Gotovushë	N	1,130	X=0506305 Y=4675861

N North; NE North-East

phytohormones cytokinin 6-benzylaminopurine (BAP) 0.5 mg l⁻¹ and auxin indole-3-acetic acid (IAA) 0.5 mg l⁻¹; **b**) WPM medium (Lloyd and McCown 1980) with BAP 0.1 and IAA 0.1 mg l⁻¹; and **c**) RA medium (40 % MS) (Tóth et al. 2004) with BAP 0.1 and IAA 0.1 mg l⁻¹. After a month, the developed plantlets were transferred to fresh media for further multiplication. Measurements of number of shoots and roots, leaves number and plantlets highest were taken.

Stage 3 (Subcultures)

Subcultures were realized in JG-B medium with BAP and IAA (0.5 mg l⁻¹ each), WPM and RA with BAP and IAA (0.1 mg l⁻¹ each) for each nutrient media.

Indirect organogenesis The pieces of the leaves and petioles in the juvenile stage of in vitro plantlets were inoculated in MS medium (Murashige and Skoog 1962) with auxins (NAA - 1-naphthaleneacetic acid) and cytokinin (BAP). For stage 1 and 2 of indirect organogenesis (Callogenesis and Meristemoid formation, respectively), explants were inoculated in MS medium with BAP 0.4 and NAA 0.5 mg l⁻¹. After meristemoid formation (Stage 3–regeneration of the organs), callus inoculated in MS medium supplemented with a high ratio of cytokinin/auxin (BAP 0.5 and NAA 0.4 mg l⁻¹).

Stage 4 (Plantlet Acclimation)

In vitro produced plantlets were kept in small containers with a mixture of soil, peat and perlite (2:1:1) (v/v) in plastic pots of 7 cm diameter and placed in a controlled growth chamber at 20 °C. From 3 to 5 plantlets from all populations of both *Ramonda* species were acclimated. The plantlets were covered with plastic bags in order to maintain

high humidity by removing them periodically for some minutes day after day. The bags were removed completely after about 4 weeks.

In vitro chamber conditions

All cultures were grown in the growth chamber at temperatures of 25 ±2 °C in a 16/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m⁻² s⁻¹.

In vitro conservation methods

For this method we applied two different methods of minimal growth:

- Reduced temperature and light intensity**—plantlets after second stage of micropropagation were transferred in JG-B medium with BAP and IAA (0.5 mg l⁻¹ each), like during the second and third stage of micropropagation. Then they were held for about 2 weeks in a vegetative room in normal conditions. After this period, the seedlings were transferred into complete dark conditions and the reduction of temperature in refrigerator (4 °C).
- Reduced JG-B salts concentration and carbohydrate**—similar to previous method plantlets from second stage of micropropagation were transferred in JG-B medium with some modifications. In this case, JG-B medium was supplemented with reduced amounts of Fe-EDTA (8.6 mg l⁻¹) and ammonium nitrate (825 mg l⁻¹) and vitamins, mio-inositol and phytohormones were not added at all to the medium. Sucrose was replaced with glucose and 0.56 % of agar was added. Plantlets were grown in the growth chamber at a temperature of 25 ±2 °C in a 16/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m⁻² s⁻¹.

The survival percentage for both methods was calculated after transferring the survived plantlets to fresh media. In this case, to calculate the survival percentage plantlets, we counted plantlets which have preserved and the ones which have lost the ability of growth and development.

Data analysis

The experiment was performed in a randomized design with three replicates. Differences among parameters and between the populations were tested using SPSS 17 statistical program. Statistical variance analysis of the all data was performed using one-way ANOVA and compared with Duncan's Multiple Range Tests at the 5 % level of significance.

Results and discussion

Distribution and ecological conditions of genus *Ramonda* populations in Kosovo

Almost in all the researched populations of *R. serbica* and *R. nathaliae* in Kosovo, they have been found to grow in gorges, in rocks and in the cracks of limestone's except for *R. serbica* in the Radac populations on rocks of dolomite. *R. serbica* grow mainly on foothills (530 m a.s.l.–Gorge of River Prizren), sometimes reaching the subalpine belts (1,651 m a.s.l.–Guri i Dellocit), while *R. nathaliae* in Kosovo grows from 982 to 1,130 m above sea level and only in Sharri Mountains (between border with Macedonia). Distribution for the most populations of both *Ramonda* species are northern and north-eastern (Table 1). Based on the obtained results during expedition *R. nathaliae* plant in Kosovo is very rare and fragmented with very lower density compared with *R. serbica* populations. Out of conducted researches throughout the expeditions we have stated that the plant community mostly dominant of populations for *R. serbica* is *Musco-Ramondaetum serbicae* Jankovic and Stevanovic (1980). In this case, the populations which had higher quantity of mosses the regeneration of that population was higher, and the populations which had lower quantity of mosses or where is not dominant the plant community with mosses, the regeneration was lower. Previous investigation reported that *R. serbica* populations in Kosovo is rare and have been threatened in category of Vulnerable (VU) plant species (Gashi 2013; Gashi et al. 2013b; Millaku et al. 2013), while *R. nathaliae* populations in Kosovo is Endangered (EN) plant species (Millaku et al., 2013). Having this fact, we have instantly started to apply methods for their micro propagation and in vitro conservation, by this we have tried to give our contribution on their ex situ preservation at germ plasm of these plants species.

Germination tests

Based on the germination test in Petri dishes, seed viability of *R. serbica* populations from Kosovo varies from 5 to 25 % (data not showed), while at *R. nathaliae* germination seeds varies from 10 to 35 % (data not showed). The maximal value was estimated for the seeds of *R. serbica* from the Gorge of river Sushica populations and Gotovusha populations for *R. nathaliae*. Two small green leaves were emerged after 10–12 days, while root tips are appeared later. According to literature (Byrne 2007), propagation is by seed sown in the spring, but the very tiny seedlings are slow to reach flowering size. Some authors (Sabovljevic et al. 2008; Gashi et al. 2012) present the data about a very low frequency of seed germination of *R. serbica* and *R. nathaliae*. In our case, weak structures of new seedlings do not allow further development into normal plants. This is the reason for the realization of the germination stage in in vitro culture, as the nutrient medium creates optimal conditions required for oligotrophic seeds of *Ramonda* plants. The higher percentage of seed germination of *R. serbica* (about 85 %) and *R. nathaliae* (about 55 %) was conducted after pre-treatment with 0.3 % KNO₃ and 500 ppm with GA₃ (data not showed). Similar results have reported from Gashi et al., 2012 for seeds of both *Ramonda* species. This pre-treatment was applied for seeds from the populations with lower germination before inoculate in the in vitro culture.

Direct organogenesis

Stage 1 (Seed germination)

Seeds from capsule septicide of *R. serbica* (Fig. 1a) and *R. nathaliae* from all populations after disinfection with ethanol 70 % were inoculated and germination in the test tube with JG-B medium without phytohormones (Fig. 1b).

Some differences for the initiation and the dynamics of seed germination of *R. serbica* and *R. nathaliae* were observed. Almost for all populations of *R. serbica* seeds start to germinate in 12th day in in vitro culture, while for *R. nathaliae* seeds start to germination in the 10th day. Similar with previous results, in this stage was obtained a little difference for germination seeds of *R. serbica* and *R. nathaliae* between populations (Fig. 2). During first stage of micropropagation in JG-B medium, the highest percentage of seed germination at *R. serbica* was observed at seeds from Gorge of river Sushica (25 %) populations, while for seeds of *R. nathaliae* from Gotovusha (34.5 %) populations (Fig. 2). Seeds germination in in vitro culture was higher at *R. nathaliae* compare to seeds of *R. serbica* (Fig. 2).

Experimental results showed that the JG-B medium without phytohormones is optimal for *Ramonda*

Fig. 1 **a** *R. serbica* plants with ripe seed capsules (Radac population); **b** Inoculated seeds in test tube with JG-B medium; and **c** Plantlets with two green leaves immediately after germination

plants in the first stage of micropropagation. This medium responds to the specific characteristics of *Ramonda* plants growth (fine and oligotrophic seeds germinating in the poor soils in their original stands). JG-B medium is especially low in chloride (instead of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, only a small amount of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ is used). The $\text{K}^+/\text{Ca}^{2+}$ amounts ratio is high. According to some literature (Tóth 2009; Sabovljevic et al. 2008; Djilianov et al. 2005), a slow metabolism and ontogenesis of the other members of *Alpin Gesneriads*, *Haberlea rhodopensis* and *Ramonda myconi* could be a consequence of the growth adaptation to the natural habitats of the plant. These species like our *Ramonda* species are restricted to shady areas with scarce mossy and thin soil layers on calcareous rocks. This slower development environment results in an alternative, but successful life strategy and does not require high concentrations of nutrients during tissue culture. Therefore these authors used MS salts only in 40 % concentration in *Ramonda myconi* in vitro cultures, justified by the results of the experiments during optimisation. It appears that the phytohormones are not necessary for seed germination medium for oligotrophic seeds of *Ramonda* plants. The same results were obtained from the experiments carried out with *R. serbica* seeds of Bulgarian area (Dontcheva et al. 2009; Daskalova et al. 2012). The authors have observed a normal organogenesis of individual plantlets from germinated seeds in the nutrient medium GM without

sugar and phytohormones. An interesting and specific fact is the positive effect of continuous irradiance on the germination as result of positively photoblastic seeds of *Ramonda* plants (Stefanovic and Neskovic 1977).

After 10–12 days of germination, seeds in test tube with nutrient media JG-B and without phytohormones, developed plantlets with two small leaves (Fig. 1c). Afterwards, the developed plantlets were separated from each other and transferred of the next stage of micropropagation.

Stage 2 (Seedling development)

Plantlets from previous stage for each species and populations were developed in the three different nutrient media: **a**) JG-B (Jungnickel/Gliemeroth Basal) medium (Jungnickel 1988) with phytohormones BAP 0.5 and IAA 0.5 mg l^{-1} ; **b**) WPM medium (Lloyd and McCown 1980) with BAP 0.1 and IAA 0.1 mg l^{-1} ; and **c**) RA medium (40 % MS) (Tóth et al. 2004) with BAP 0.1 and IAA 0.1 mg l^{-1} . In this stage of micropropagation, some differences were observed between plantlets from different populations and nutrient media. Differences in the development of plantlets between *R. serbica* and *R. nathaliae* were also observed (Tables 2 and 3). Based on the results (Table 2) for *R. serbica*, the highest number of shoot, roots, leaves and plantlets highest was recorded at plantlets from origin of Gorge of river Sushica in nutrient media JG-B with BAP 0.5 and IAA 0.5 mg l^{-1} ; similar

Fig. 2 Germination percentage of seeds of *R. serbica* and *R. nathaliae* plants from different populations in JG-B nutrient media. Number from 1 to 11 are from Table 1 how indicated the code of populations

results were recorded also for plantlets from Radac populations. Among the three nutrient media, the best nutrient media for this stage of micropropagation for highest number of shoots and roots was JG-B, while for highest number of leaves and plantlets highest was WPM medium with BAP 0.1 and IAA 0.1 mg l⁻¹ (Table 2). The highest number of shoots was observed for plantlets from origin of Canyon of Rugova in nutrient media WPM (4.95), while number of roots was higher (3.00) at Gorge of river Sushica in nutrient media JG-B and number of leaves (18.43) in nutrient media WPM (Table 2). The highest plantlets of *R. serbica* was in Shkëmbi i përgjakur populations in nutrient media WPM (30.46) (Table 2). Almost for all population of *R. serbica* and *R. nathaliae* plantlets, the poor results were observed in the nutrient media RA with BAP 0.1 and IAA 0.1 mg l⁻¹ (Table 2). For all researched parameters of micropropagation during second stage and in three nutrient media, plantlets of *R. nathaliae* from Gotovusha populations were higher than Gllboçica populations (Table 2). Based on Duncan test for number of shoots and highest number of plantlets, the effect of JG-B medium in development of plantlets of *R. serbica* from different populations has not significant effect between populations, while for RA medium was conducted high significant differences between populations for number of roots (Table 2).

In Table 3 are shown the results of analysis of evaluation variance of populations and nutrient media on studied parameters during second stage of micropropagation of *R. serbica* and *R. nathaliae* plantlets originated from different populations. Mean value of all nutrient media for all plantlets of different populations of *R. serbica* have significant effects on the number of shoots and roots, while for *R. nathaliae* have not effect. For all researched parameters during this stage of micropropagation mean value of all plantlets of *R. serbica* from different populations, nutrient media have significant differences. Also at plantlets of *R. serbica* have found some interaction between populations and nutrient media for number of roots during development of plantlets, but nor for other parameters and other plant species (Table 3).

Several nutrient media of *R. serbica* and *R. nathaliae* plants development of different variants of in vitro culture appeared:

- Normal organogenesis of a typical single plant of Ramondas with the leaves in rosette and

rapid promotion of axillary bud formation and the development of plantlet bundles in nutrient media JG-B with BAP and IAA (0.5 mg l⁻¹ each) (Fig. 3a). Cytokinin BAP appears to break the dormancy of buds of Ramonda plants, while the presence of auxin IAA induces the rhizogenesis of the plantlets (Fig. 3b);

- Slow development of plantlets and some modifications in the leaves structure (hyper hydration) in the nutrient media RA (Fig. c) with BAP and IAA (0.1 mg l⁻¹ each).
- Rapid development of plantlets and highest plantlets, but a little modification in typical morphology of ramnoda leaves (Fig. 3d) in the nutrient media WPM with BAP and IAA (0.1 mg l⁻¹ each).

Stage 3 (Subcultures)

Plantlets of *R. serbica* and *R. nathaliae* from previous stage were used for subcultures. All plantlets of both species from different populations were transferred in the three nutrient media similar with second stage. Axillary buds of plantlets were separated and inoculated in each test tube with different nutrient media. The axillary bud method was especially successful for rosette plants. The newly developed shoots formed from the axillary buds in the leaf axil under the influence of the cytokinin dose were transferred to fresh medium, on which axillary shoot formation took place again (Fig. 4).

Results of the multiplication rate after the first subculture of the plantlets originated from different populations and different nutrient media for both Ramonda species are shown in Fig. 5. The highest multiplication rate of plantlets of *R. serbica* was recorded at Gorge of river Sushica populations (7.25) and at Shkëmbi i përgjakur populations (6.40) in nutrient media JG-B with BAP and IAA (0.5 mg l⁻¹, each). On other hand, in all of nutrient media the lowest multiplication rate was recorded at Radaci and Gorge of Rusenica populations. Almost for all plantlets originated from different populations of both species, the best nutrient media for highest number of plantlets after first subculturing was JG-B with BAP and IAA (0.5 mg l⁻¹, each) and WPM with BAP and IAA (0.1 mg l⁻¹, each) (Fig. 5). Similar results were conducted also for plantlets of *R. nathaliae*. This procedure is decisive to produce a greater number of plants during a period of 4 months.

Table 2 Effects of nutrient media and plantlets origin from different populations of *R. serbica* and *R. nathaliae* on studied parameters during stage one of micropropagation

	Nutrient media Populations	Number of shoots			Number of roots			Number of leaves			Plantlets highest		
		JG-B			JG-B			JG-B			JG-B		
		RA	WPM	RA	WPM	RA	WPM	RA	WPM	RA	WPM	RA	WPM
<i>R. serbica</i>	Gorge of Zhlebi	3.74 ^A	2.58 ^B	2.92 ^B	1.30 ^{BC}	1.13 ^B	1.36 ^{BC}	7.77 ^B	10.35 ^A	10.76 ^B	18.97 ^A	22.66 ^A	23.51 ^{BC}
	Radac	4.20 ^A	3.65 ^{AB}	3.83 ^{AB}	2.43 ^{AB}	1.80 ^B	1.41 ^{BC}	13.83 ^A	11.61 ^A	12.04 ^{AB}	23.11 ^A	24.25 ^A	25.57 ^{A-C}
	Gorge river of Sushica	4.49 ^A	3.60 ^{AB}	4.12 ^{AB}	3.00 ^A	1.70 ^B	2.26 ^A	14.21 ^A	13.44 ^A	18.43 ^A	24.08 ^A	24.54 ^A	31.21 ^A
	Canyon of Rugova	4.05 ^A	4.62 ^A	4.95 ^A	2.13 ^{A-C}	1.93 ^{BC}	2.03 ^{AB}	9.06 ^B	12.06 ^A	15.41 ^{AB}	18.45 ^A	23.89 ^A	27.68 ^{A-C}
	Gorge of Koprivnik	4.04 ^A	3.65 ^{AB}	3.99 ^{AB}	2.23 ^{A-C}	2.96 ^A	1.53 ^{A-C}	9.17 ^B	13.22 ^A	14.10 ^{AB}	20.77 ^A	25.29 ^A	26.54 ^{A-C}
	Gorge of river Prizren	3.73 ^A	2.99 ^B	3.17 ^B	1.46 ^{BC}	2.70 ^{AB}	1.36 ^{BC}	9.40 ^B	11.09 ^A	11.50 ^{AB}	19.24 ^A	21.52 ^A	23.19 ^{BC}
	Gorge of Rusenica	4.30 ^A	3.26 ^B	3.47 ^B	2.30 ^{A-C}	1.63 ^B	1.36 ^{BC}	9.87 ^B	10.63 ^A	11.11 ^B	21.99 ^A	20.88 ^A	22.27 ^C
	Gorge of Matosi	4.01 ^A	3.64 ^{AB}	3.96 ^{AB}	2.36 ^{AB}	1.06 ^B	1.13 ^C	9.88 ^B	11.44 ^A	12.26 ^{AB}	20.73 ^A	24.51 ^A	25.61 ^{A-C}
	Shkëmbi I përjakur	4.26 ^A	3.69 ^{AB}	4.01 ^{AB}	2.53 ^A	1.40 ^B	1.43 ^{BC}	9.39 ^B	13.26 ^A	17.06 ^{AB}	21.01 ^A	25.65 ^A	30.46 ^{AB}
	Mean	4.09	3.52	3.82	2.19	1.81	1.54	10.29	11.90	13.63	20.93	23.69	26.23
<i>R. nathaliae</i>	Significance	0.655	0.020	0.021	0.02	0.000	0.078	0.12	0.950	0.127	0.555	0.741	0.102
	Gllloboçica	3.39	3.29	3.83	2.30	1.36	1.69	8.46	10.03	12.54	18.55	24.07	28.47
	Gotovusha	4.52	4.00	4.77	2.00	1.43	1.33	12.81	12.81	16.54	23.48	22.86	30.88
	Mean	3.96	3.65	4.30	2.15	1.40	1.51	10.64	11.42	14.54	21.02	23.47	29.67
	Significance	0.060	0.232	0.139	0.552	0.439	0.247	0.113	0.777	0.521	0.193	0.813	0.256

JG-B Jungnickel/Gliemeroth Basal) medium (Jungnickel, 1988) with phytohormones cytokinin 6-benzylaminopurine (BAP) 0.5 mg l⁻¹ and auxin indole-3-acetic acid (IAA) 0.5 mg l⁻¹; WPM medium (Lloyd and McCown, 1980) with BAP 0.1 mg l⁻¹ and IAA 0.1 mg l⁻¹; and RA medium (40 % MS) (Tóth et al., 2004) with BAP 0.1 mg l⁻¹ and IAA 0.1 mg l⁻¹. Mean in each column followed by same letters are not significantly different at the P 0.05 by one-way ANOVA with Duncan's multiple range tests

Table 3 Analysis of evaluation variance of populations and nutrient media on studied parameters during micropropagation of *R. serbica* and *R. nathaliae* plantlets

Source	Dependent variable	Sum of squares	Df	Mean square	F	Sig.
<i>R. serbica</i>						
Populations	No. of shoots	39.600	8	4.950	4.632	0.000
	No. of roots	23.530	8	2.941	3.744	0.000
	No. of leaves	628.506	8	78.563	2.563	0.011
	Highest number of plantlets	722.511	8	90.314	2.148	0.033
Nutrient media	No. of shoots	13.276	2	6.638	6.212	0.002
	No. of roots	23.530	8	2.941	3.744	0.000
	No. of leaves	452.149	2	226.074	7.375	0.001
	Highest number of plantlets	1138.779	2	569.390	13.545	0.000
Populations * Nutrient media	No. of shoots	12.981	16	0.811	0.759	0.730
	No. of roots	17.339	2	8.669	11.037	0.000
	No. of leaves	375.676	16	23.480	0.766	0.723
	Highest number of plantlets	434.110	16	27.132	0.645	0.844
<i>R. nathaliae</i>						
Populations	No. of shoots	11.491	1	11.491	7.716	0.008
	No. of roots	0.520	1	0.520	0.842	0.363
	No. of leaves	185.704	1	185.704	4.110	0.048
	Highest number of plantlets	56.202	1	56.202	0.846	0.362
Nutrient media	No. of shoots	3.836	2	1.918	1.288	0.285
	No. of roots	5.898	2	2.949	4.774	0.013
	No. of leaves	153.473	2	76.736	1.698	0.194
	Highest number of plantlets	717.175	2	358.587	5.396	0.008
Populations * Nutrient media	No. of shoots	0.406	2	0.203	0.136	0.873
	No. of roots	0.474	2	0.237	0.383	0.684
	No. of leaves	6.041	2	3.020	0.067	0.935
	Highest number of plantlets	85.797	2	42.898	0.645	0.529

Same letters are not significantly different at the P 0.05

Fig. 3 **a** and **b** *R. serbica* plantlet in JG-B medium with BAP and IAA (0.5 mg l⁻¹, each); **c** *R. serbica* plantlets in RA medium with BAP and IAA (0.1 mg l⁻¹, each); **d** *R. serbica* plantlets in WPM medium with BAP and IAA (0.1 mg l⁻¹, each)

Fig. 4 *R. serbica* plantlets in bundles, ready to the subculture. Foto by E. Kongjika

Indirect organogenesis

The results for indirect organogenesis were very similar for *R. serbica* and *R. nathaliae* plants from different populations. For this method of micropropagation, the material used were pieces of leaves and petioles from native plant of both species (from natural habitat of both species) and from pieces of leaves and petioles of plantlets after subculture (plant material from in vitro culture). There were severe problems to obtain in vitro cultures starting from leaves and petioles with origin from plant material of natural conditions, because of the unusual morphology of *R. serbica* and *R. nathaliae* leaves. To obtain axenic culture from collected plant material from natural condition was impossible because of different methods of sterilization of pieces of leaves and petioles

showed no satisfactory results, because the high concentrations of solvents damage the plant material, while the low concentrations have not effect for sterilization. The negative result for micropropagation of *R. serbica* from natural plant material also was reported from Sabovljevic et al. 2008. On other hand, the axenic culture from pieces of leaves and petioles of plantlets after subculture was successful for both *Ramonda* species.

First stage (Callogenesis) and second stage (Meristemoid formation) during indirect organogenesis from pieces of leaves and petioles of plantlets after subculture for both *Ramonda* species and all populations, was realised in combination of MS medium with BAP 0.4 and NAA 0.5 mg l⁻¹. First initiation of callus was visible within 2 weeks at cut edges of leaf pieces (Fig. 6a). Calli were formed along the microinjuries and

Fig. 5 Multiplication rate after first subculturing of *R. serbica* and *R. nathaliae* plantlets from different populations (number 1–11 indicate the code of populations from Table 1) in nutrient media: JG-B with BAP and IAA (0.5 mg l⁻¹, each), WPM with BAP and IAA (0.1 mg l⁻¹, each) and RA with BAP and IAA (0.1 mg l⁻¹, each)

Fig. 6 Indirect organogenesis of *R. serbica* plantlets from leaf pieces: **a** Callus formation, **b** dedifferentiated callus cells, **c** Meristemoid formation from callus, and **d** New plantlet of *R. serbica* derived from the indirect organogenesis

dedifferentiated callus cells (Fig. 6b) were derived from the proliferation of basal somatic cells of the explants in contact with nutrient medium supplemented with phytohormones in high ratio auxin/cytokinin. Developed meristemoid centres (Fig. 6c) were derived from the parenchymatic cells of the callus. It seems that meristemoid shape of two *Ramonda* species is very similar. The development of visible meristemoids with regenerative potential is followed by cell differentiation and the formation of new plantlets with the leaves in rosette (Fig. 9). Third stage of indirect organogenesis (Organ formation) was very similar for all populations of both *Ramonda* plantlets. The MS medium supplemented with a high ratio of cytokinin/auxin (BAP 0.5 mg l^{-1} and NAA 0.4 mg l^{-1}) has stimulated the regeneration of new organs (Fig. 6d) from the development of the meristemoids. The callus initiation, the meristemoid formation and the regeneration of adventitious organs of two *Ramonda* species are very similar models. According to the literature (Sabovljevic et al. 2008), there is a remarkable similarity between *R. serbica*, *R. myconi* and *Haberlea rhodopensis* in all important aspects of in vitro culture, starting from leaves and petioles.

Stage 4 (Plantlet acclimation)

In vitro produced plants of *R. serbica* and *R. nathaliae* were kept in small containers with a mixture of soil, peat and perlite (2:1:1) (v/v), in plastic pots of 7 cm diameter and placed in a controlled growth chamber at 20°C . The plantlets were covered with plastic bags (3–5 plantlets per populations) in order to maintain high humidity by removing them periodically for some minutes day after day. The bags were removed completely after about 4 weeks. Acclimated plantlets (Fig. 7) may be used for different destinations.

In vitro conservation

For in vitro conservation, as initial plant material of *R. serbica* and *R. nathaliae* have been used the plantlets from second stage of micropropagation (after subculture) in JG-B nutrient media with phytohormone. In vitro conservation was realised with plantlets originated from different populations of both

Ramonda species from Kosovo. The diversity of germplasm of these plant species from different populations it's very important for preservation and protect of these rare and endemic plant species, and also these plant materials can be used for further investigation. For in vitro germplasm preservation of both *Ramonda* plantlets we applied two different methods with minimal growth, plantlets were incubated in reduced temperature and light intensity and in reduced JG-B salts concentration and carbohydrate.

The effects of reduced temperature and light regime in stored plantlets material of both *Ramonda* species were realised to cultivation of plantlets in nutrient media JG-B with BAP and IAA (0.5 mg l^{-1} each). For developing slow growth cultures with this method, firstly, the culture temperature was optimized by keeping the cultures at vegetative room in normal conditions for 2 weeks. After this step, plantlets were transferred in refrigerator (4°C) and complete darkness conditions. The tests of survival ability of plantlets for regeneration after stored in these conditions were made in three different periods (4, 8 and 12 months). The survival percentage is calculated after transferring the survived plantlets in the fresh media. From the obtained result from this method (Fig. 8) for both *Ramonda* species, showed that the maximum storability was determined to be 12 months. Almost for all plantlets of both *Ramonda* species from different populations with this method can be stored in optimal period for 6 month (Fig. 8). The highest survival percentage for 4 months showed plantlets originated from Radaci and Gorge of Sushica populations for *R. serbica*, and for *R. nathaliae*, plantlets from Gotovusha populations (Fig. 8). Some differences between populations for survival percentage were conducted for plantlets from Gorge of Rusenica and Gorge of Matosi, which have minimal survival percentage compare with other populations of plantlets of *R. serbica* (Fig. 8). These observations were made on the basis of visual morphological changes. Although, the plantlets incubated at such a low temperature and light intensity turned yellowish in color, leaf size became very small but the shoot tips were quite fresh and green (Ahmed et al. 2010). Catana et al. (2010) reported that the in vitro conservation of plantlets of *Gypsophila petraea* and *Dianthus callizonus* in temperature 10°C can be survived for 12 months. Decline in incubation temperature proved to be very effective in slow growth

Fig. 7 Acclimated plants of *R. serbica*

conservation strategy because it enhances subculture period (Divakaran et al. 2006; Engelmann 2011).

The effects of reduced JG-B salts concentration and carbohydrate in stored plantlets material of both *Ramonda* species were realised to cultivation of plantlets in nutrient media JG-B without phytohormone and with some modification of salt concentration. In this case, JG-B medium was supplemented with reduced amounts of Fe-EDTA (8.6 mg l^{-1}) and ammonium nitrate (825 mg l^{-1}) and no vitamins, mio-inositol and

phytohormones were added. Sucrose was replaced with glucose and 0.56 % agar was added.

Plantlets were grown in the growth chamber at temperatures of $25 \pm 2^\circ \text{C}$ in a 16/8 h light/dark regime with cool, white fluorescent light of intensity $43.4 \mu\text{mol m}^{-2} \text{s}^{-1}$. Also for this method, the 3test of survival ability of plantlets for regeneration after stored in these conditions was made in three different periods (3, 5 and 6 months). The survival percentage was calculated after transferring the survived plantlets in the fresh media.

Fig. 8 Survival percentage of *R. serbica* and *R. nathaliae* plantlets from different populations (number 1–11 indicate the code of populations from Table 1), conserved in reduced temperature and light regime

Fig. 9 Survival percentage of *R. serbica* and *R. nathaliae* plantlets from different populations (number 1–11 indicate the code of populations from Table 1), conserved in nutrient media with reduced JG-B salts concentration and carbohydrate

The experimental data obtained from this conservation method (Fig. 9), showed that the plantlets conserved for a maximum periods of 6 months. For all plantlets originated from different populations of *R. serbica* and *R. nathaliae*, the optimal storability by this method was 5 months (Fig. 9). Survival percentage of conservation for periods of 3 months varies from 40 to more than 90 % at *R. serbica*, while for *R. nathaliae*, this period and latest period survival percentage was higher than *R. serbica* (Fig. 9). Similar with previous method of conservation, the highest survival percentage for *R. serbica* was observed for plantlets originated from Gorge of river Sushica and Radaci populations (Fig. 9). Our results for this method are in accordance with the results from other authors. Ivanova et al. (2011) have reported that the plantlets of *Ruscus aculeatus* can be preserved for 6 months in the nutrient media without phytohormones and in temperature at 24 ± 1 °C. Reduction of salt concentrations and elimination or reductions of sucrose in nutrient media have significant effect on the in vitro conservation of other plant species (Catana et al. 2010; Holobiuc et al. 2006, 2008, 2009). Similar results about in vitro conservation with modification of nutrient media and supplement, reduced temperature and low light intensity was reported by other authors (Moges et al. 2003; Shibli et al. 2006).

Conclusions

The success of micropropagation and in vitro conservation of *R. serbica* and *R. nathaliae* plants depends on the optimal choice of the explants and on the efficiency of the

sterilization method. The most important step for the establishment of an in vitro culture protocol for these species is the initiation of aseptic proliferative cultures and to choose the optimal nutrient media. After this step, the multiplied material could be maintained in vitro, cryo-preserved or used in restoration programs.

Based on our experimental data, we came up to these conclusions:

- Micropropagation of *R. serbica* and *R. nathaliae* plants from different populations from Kosovo is very important to help the conservation of these species germplasm and to reintroduce the micropropagated and acclimated plants in regions where the specimens are few in number.
- The optimal medium used during direct organogenesis is considered to be JG-B medium, while during indirect organogenesis universal MS medium. This method may be used also for other spontaneous, rare, endemic and threatened plant species from Kosovo.
- Nutrient media WPM with phytohormones IAA and BAP (0.1 mg l^{-1} , each) have effect in rapid development of plantlets and highest plantlets, but a little modification in typical morphology of Ramonda leaves.
- The best plant material of *R. serbica* with significant results for micropropagation and in vitro conservation are plantlets originated from Gorge of river Sushica populations, while for *R. nathaliae* from Gotovusha populations.
- The optimal method for in vitro conservation of plant material of both Ramonda species for maximum long time preservation (12 months) is in nutrient media JG-B with

BAP and IAA (0.5 mg l^{-1} each) and incubated in reduced temperature at 4°C and in complete darkness conditions.

- Micropropagation of *Ramonda* plants will be used to create an *in vitro* gene bank for germplasm conservation of the Balkanic Gesneriads (*Ramonda serbica*, *R. nathaliae*, *Jankaia heldraichii* and *Haberlea rhodopensis*). Our experimental data and the data from other authors show that there is a great similarity between these species in all important aspects of *in vitro* culture.

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