

Some considerations regarding the *In Vitro* culture of *Rhodiola rosea* L.

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Abstract

In vitro cultures of *Rhodiola rosea* were initiated and the morphogenetic reaction of several types of explants was tested in view of elaborating a micropropagation technique for this species, to provide *in vitro* regenerants for repopulating native habitats of *R. rosea*, where it has extinguished or is endangered. It was ascertained that the most efficient variants of MS medium to provide neoplantlets from shoot nodes and apices are: N (2.0 mg l⁻¹ NAA), followed by hormone free MS, then KN (1 mg l⁻¹ Kin + 0.5 mg l⁻¹ NAA), and AZ (0.2 mg l⁻¹ IAA + 2 mg l⁻¹ zeatin). The *in vitro* regenerants transfer on Ceahlău Mountains (at 1750 meters altitude) proved that the achievement of our goal is possible.

Keywords: *Rhodiola rosea*, *in vitro* culture, morphogenetic reaction, micropropagation

Introduction

Rhodiola rosea L. is a dioecious, perennial plant, and belongs to the *Crassulaceae* family. This species was initially included in the *Sedum* genus, but in 1963 HEGI (quoted by Gemano et al., 1999) separated the *Rhodiola* group (comprising about 50 species) as a distinct item of this genus.

There are about 200 species within *Rhodiola* genus [1]. The genus *Rhodiola* probably originated in the mountainous regions of Southwest China and the Himalayas [2]. The species of the *Rhodiola* genus have a circumpolar Northern distribution and in the mountain regions of Europe (from Iceland and Scandinavia to Pirinei, Alpes, Carpathians and Balkans). LINNÉ named the species *rosea* due its root scent of rose. This plant has been used since antiquity as a remedy for numerous health disorders and it is well known in the traditional medicine for the benefits of its root extracts: improvement of physical condition, treatment of anemia, depression, asthenia, impotence, scurvy, gastro-intestinal and nervous system disorders and also as a stimulant and anti-inflammatory, [2 - 4]. Phytochemical investigations effected on *Rhodiola rosea* rhizomes and roots evinced six different groups of important pharmaceutical substances: phenylpropanoids, phenyl-ethanolic derivates, flavonoids, monoterpenes, triterpenes and phenolic acids, [2, 3, 5, 6]. Modern phytotherapy considered this species a vegetal source with an antioxidant and antistress-adaptogene action [3, 7 - 9], due to influence of some of its compounds on the level of monoamine and peptide (β -endorphine type) in the human body [4]. This action is mainly due to rosavine and salidroside. There is a growing interest to produce some of these compounds in *in vitro* cultures of *R. rosea* [2, 10].

In Romania, this species was found in the mountains of Rarău, Ceahlău, Bucegi, Călimani, Rodnei, Făgăraș, Maramureș, etc. We have no information regarding the use of *Rhodiola rosea* roots in Romania for some pharmaceutical preparations, either by disregard of therapeutic importance of this species, or due to its areal (high altitude, less accessible regions). Nevertheless, this species is no longer found in some natural habitats where it used to grow (Rarău Mountains, e.g.) or the number of individuals is extremely low (Ceahlău Mountains). Therefore, we proceeded with a challenging aim: to test the *in vitro* morphogenetic reaction of *Rhodiola rosea* L. and to elaborate an *in vitro* micropropagation technique for subsequently repopulate the natural habitat of this species in Ceahlău

Mountains. The fact that some researchers succeeded in cultivating *R. rosea in vitro* and provided callus or regenerants [5, 11 - 13], proves that this aim can be achieved.

Material and methods

The biological material to initiate the *in vitro* cultures of *Rhodiola rosea* was part of a population from Ceahlău Mountains, at about 1750 meters altitude. The explants were vegetative buds harvested from rhizomes during autumn (the months of September and October), or apices and shoot nodes harvested from young plants of the original population, from individuals transferred into culture at “Stejarul” Research Centre in Piatra Neamt, and also from lab-cultivated plants (under specific conditions) during spring. After a series of unsuccessful tests, it was only this latest explant source that provided sterile and viable explants (short – term immersion in 5% chloramine-T solution). The nutritional medium was Murashige-Skoog (1962), comprising sucrose (25 g l⁻¹) and agar-agar (8.5 g l⁻¹) to solidify it. The explants (apices and shoot nodes with leaves) were cultivated in Erlenmeyer vials (B-type) on hormone-free MS medium, on MS enriched with BAP (0.2 mg l⁻¹) or with kinetin and NAA (1 and 0.5 mg l⁻¹, respectively). In order to resume growth processes the inoculated vials were placed in a climatized incubator at the University of Bacău (temperature 20°C, illumination 2000 lux, photoperiod 12 hours). The sterile neoplantlets obtained were subsequently used as an explant source to evince the *in vitro* behaviour of various types of explants of this species (apices, nodes, internode, leaf and root fragments) on several variants of basal MS culture medium. The results of our investigations are presented in Table 1 and figure 1.

Results

In vitro cultures initiation of *Rhodiola rosea* was quite a difficult task, as this species is sensitive to disinfection agents. All our attempts to initiate the *in vitro* culture using plants from their natural habitat as an explant source were unsuccessful as we were unable to induce explant sterility and maintain their viability simultaneously. Therefore we considered as a pre-requisite to find a way to reduce microbial load of explant donor plants. The period of time for explants immersion into disinfecting agents had to be shortened. In this respect, we harvested some plants from their native environment and cultivated them into soil pots (in laboratory conditions) and submitted them to low temperature. This action allowed a shorter disinfection time (using a milder agent, as chloramine-T, 5%) of only 3 to 6 minutes. Another aspect we need to specify is that *in vitro* growth processes of this species are much slower compared to some of its keen species, such as: *Sedum fabaria* or *Sedum hybridum*, [14, 15].

Though *in vitro* culture initiation of *R. rosea* was accomplished on several hormonal variants of MS medium, the best results for micropropagation action we obtained were on hormone-free MS, that provided neoplantlets with strong roots and 2-5 shoots from basal nodes, thin stems, quite small leaves. These neoplantlets reached 5 to 7 cm in height in a period of time of 2.5 months, (Table 1; Fig. 1a). The sterile shoots obtained were then used to test the morphogenetic reaction of varied explants from this species on nutritive media supplemented with growth regulators in varied combinations and concentrations.

Apices and shoot nodes cultivated on MS medium enriched with BAP (0.2 mg l⁻¹) displayed a quite poor morphogenetic reaction. Very small shoots grew and sporadically developed roots in the nutritive medium. On MS medium supplemented with two cytokinins (BAP and kinetin), the apices and nodes generated neoplantlets with several shoots but very few roots; the shoots had thin stems, rather long internodes and small leaves; there were two types of roots: short and white, or long, dark colored and with secondary branches. Media supplemented with cytokinins are not a promising perspective for *Rhodiola rosea in vitro* cultures in view of regenerants production.

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Table 1. The morphogenetic reaction of some *Rhodiola rosea* L. explants on several variants of Murashige – Skoog medium

Var.	Explant	Medium variant	Growth regulators (mg l ⁻¹)								Morphogenetic reaction and proliferation speed
			BAP	GA	IAA	IBA	NAA	KIN	2.4-D	Z	
1	Apices, nodes	A			2.0						Neoplantlets (++) with 1-4 shoots/node, longer internodes, larger leaves and well developed roots (+++)
2	“	AZ			0.2					2.0	Neoplantlets (+++) with 1-4 shoots/node, thin stems with small leaves; very intense rhizogenesis (++++) within the nutritive medium and at its surface, numerous short fascicled white roots, (some of them with negative geotropism)
3	“	B	0.2								Neoplantlets (+) with very slow growth, multiple shooting (++) , shoots with short internodes and small leaves, poor rhizogenesis (+)
4	“	BA	1.0		0.5						Compact cream callus (+), at contact surface with the nutritive medium, that provides small multiple shoots (++) , with short internodes, small leaves, short white roots with many absorbent hairs (++)
5	“	BB	1.0			1.0					Neoplantlets (+++) with multiple shoots, longer internodes, intense rhizoogenesis (+++); long, dark-colored roots
6	“	BG	1.0		0.5						Neoplantlets (++) with 1-2 shoots/node, long dark-colored roots, multiple shoots (++) , shoots with short internodes and small leaves
7	“	BK	0.5					0.5			Neoplantlets (++) , multiple shoots (+++); offshoots with thin stems, long internodes, small leaves; low rhizogenesis (+), long roots (either white or dark-coloured) with secondary branches
8	“	BN	1.0				0.5				Neoplantlets (++) , multiple shoots (+++) with short internodes, good rhizogenesis (++)
9	“	IB				2.0					Neoplantlets (+), low growth rate, frail shoots, small leaves, rhizogenesis sporadically (+)

10	“	KN					0.5	1.0		Neoplantlets (+++) with 3-7 shoots/node; very intense rhizogenesis (++++), mostly at the surface of the nutritive medium; fascicled roots (dark-colored – those within the nutritive medium, white – those from the surface)
11	“	N					2.0			Neoplantlets (+++) with 1-2 basal offshoots; more vigorous shoots, thicker stems, larger and thicker leaves; the most intense rhizogenesis (++++) – roots arranged as a white felt within the nutritive medium and at its surface
12	“	MS								Neoplantlets (+++) with 1-2 shoots from the basal node; shoots with longer internodes and larger leaves than the ones provided on media supplemented with growth regulators; regular rhizogenesis (++) – fascicled white roots, some of them display a negative geotropism
13	Internode fragments	BD	1.0						0.5	Compact green callus (++) on the entire explant surface, mostly at internode fragment ends
14	“	D							2.0	Compact cream callus (+), intense rhizogenesis (+++)
15	Leave fragments	BD	1.0						0.5	The fragments grew intensely and provided a compact, brown-greenish callus (+) and short white roots (++) within the nutritive medium and at their surface; some leaves sporadically provided shoots (+) and neoplantlets with frail stems, small leaves and roots (short and white and also long, dark-colored ones)
16	“	BA	1.0		0.5					Leaves grew intensely and provided a compact green callus (+)
17	“	D							2.0	Sporadically shoots (+), intense rhizogenesis (+++)
18	“	KN					0.5	1.0		The fragments grew and sporadically generated white fascicled roots (mostly from the petiole); small shoots seldom appeared from the compact callus provided on this medium formula
19	Root fragments	BD	1.0						0.5	Sporadically, compact cream-greenish callus (+) and shoots (+), intense rhizogenesis (+++)

+ poor reaction; ++ moderate reaction; +++ good reaction; ++++ very good reaction.

It was ascertained that by adding NAA (2 mg l^{-1}) to the nutritive medium, the nodes and shoot tips provided strong neoplantlets (Table 1), with 1-2 shoots from basal node (the shoots had a thicker stem, longer internodes and larger leaves than those generated on other hormonal variants); the presence of NAA in the nutritive medium led to the most intense rhizogenesis; a dense net of roots developed either in the nutritive medium or at its surface. In case of supplementing the culture media with another auxin (IBA), the growth processes at the level of apices and nodes were very slow and rhizogenesis appeared only sporadically. These explants displayed a similar development on MS comprising both BAP and giberellin.

The apices and shoot nodes had a more favorable morphogenetic reaction on some hormonal variants that included combinations of cytokinins and auxins. Neoplantlets (frequently with several shoots, thin stems and small leaves) were formed on MS supplemented with IAA (0.2 mg l^{-1}) and zeatin (2 mg l^{-1}). Rhizogenesis was also very intense on this hormonal variant, particularly on its surface, the roots covered the medium in a felted layer; some roots displayed a negative geotropism (Table 1). The explants placed on AZ (IAA + zeatin) medium had a rather similar reaction to the one on KN (Fig. 1c); neoplantlets with several basal shoots, less vigorous than on N and AZ media, with small leaves; rhizogenesis was very intense, the roots within nutritive medium were fascicled and dark colored, and the ones at surface appeared as a white net. On BA (1 mg l^{-1} BAP and 0.5 mg l^{-1} IAA), we noticed an extremely slow shoot growth and a poor rhizogenesis, the neoplantlets were stunted.

During our investigations we also tested the reaction of other types of explants on certain hormonal variants. We observed that internode fragments cultivated on BD, MS medium enriched with BAP (1 mg l^{-1}) and 2,4-D (0.5 mg l^{-1}), generated a compact, green callus with a more intense proliferation at its sectioned ends (Fig. 1b). At the moment we cannot appreciate its proliferation or differentiation capacity following the transfer on MS medium enriched with various growth regulators.

Leaf fragments inoculated on BD medium grew, became thicker, and generated a semi-compact cream callus on some leaf regions; in most cases they generated white roots in the nutritive medium or at its surface. Sporadically, some leaf fragments provided shoots and neoplantlets that formed either short, white roots, or long, dark-colored roots (Fig. 1d). We noticed a similar reaction on the nutritive medium KN.

Root fragments inoculated on BA grew and provided a compact, thin, low proliferative callus that has not differentiated either roots, or shoots. Root fragments inoculated on BD seldom formed a compact, low-proliferative, cream-greenish callus and secondary roots (rhizogenesis was more intense than callogenesis).

The observations on the *in vitro* morphogenetic reaction of some *Rhodiola rosea* explants proved that the most appropriate types of explants for micropropagation are shoot apices and nodes, and the most indicated medium variants of MS are: N > hormone-free MS medium > KN > AZ. DIMITROV et al. (2003) also considered the latter combination to be efficient in the process of *in vitro* micropropagation of this species, (11).

The *in vitro* generated neoplantlets were accommodated to septic environment in a hydroponic system (Fig. 1e), a quite facile process accomplished in a short period of time (5-7 days). Over 90% of the regenerants survived this stage. After acclimatization, the regenerants were placed into soil pots, where maintained until their transfer to the native habitat (Ceahlău Mountains). During the month of June 2007, a few dozens of *in vitro* regenerants of *Rhodiola rosea* were planted in the mountains.

The recurrent studies showed that during the summer of 2008 about 73.5% of the regenerants survived, while this percentage dropped at 57% during 2009. It was ascertained that the *in vitro* regenerants of *Rhodiola rosea* transferred in their natural habitat are different (Fig. 1f) in the respect of leaf color (light green), compared to the native individuals of this region (green- grey).

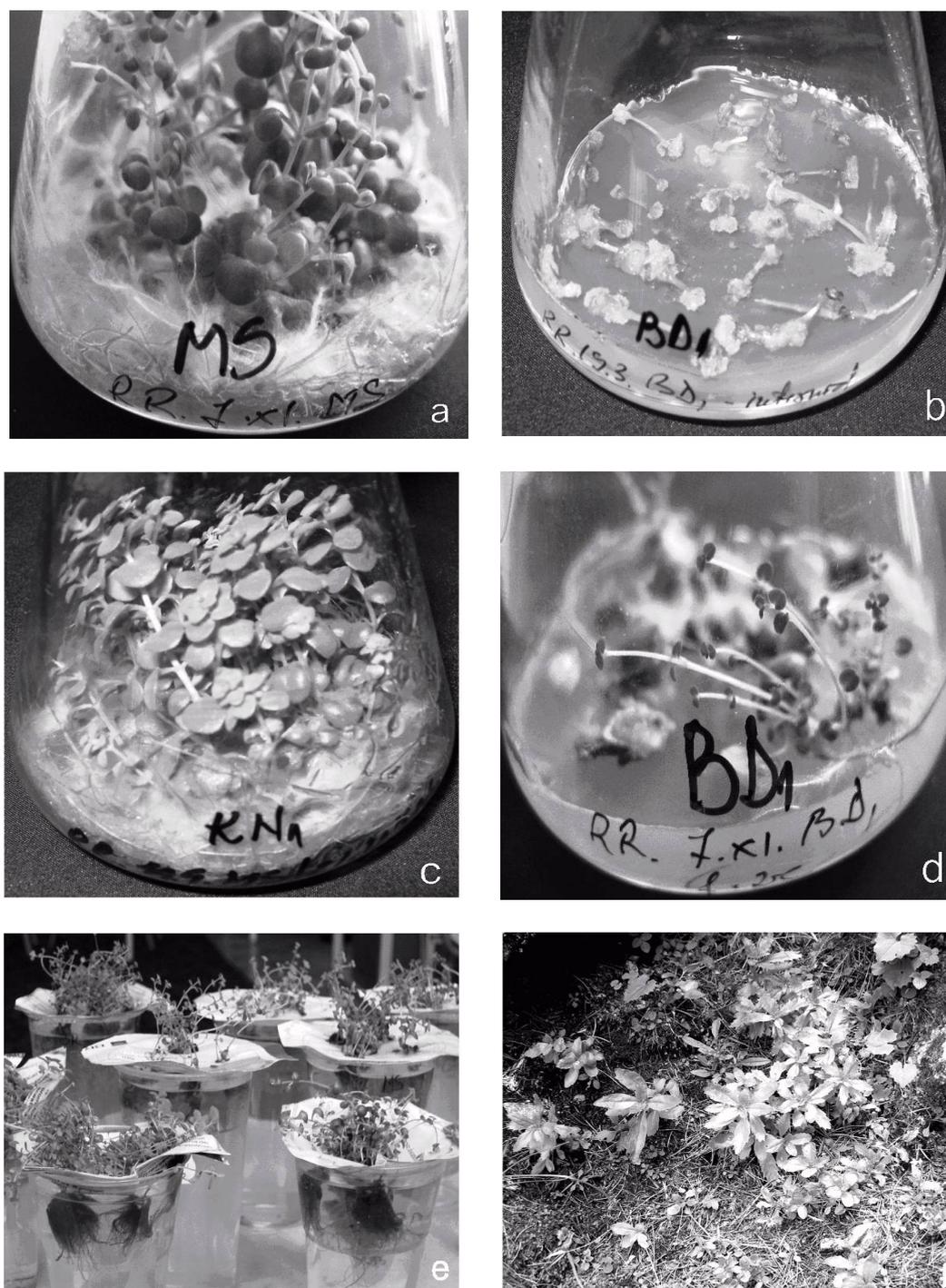


Figure 1. Aspects of *in vitro* cultures of *Rhodiola rosea* L.

a) neoplantlets on MS hormone free medium; b) callus of internodes fragments; c) neoplantlets on MS variant supplemented with kinetin and NAA; d) callus, roots and neoplantlets generated from leaf fragments; e) neoplantlets accommodation in hydroponic system; f) regenerants of *R. rosea* transplanted in the natural habitat of this species (Ceahlău Mountains) - second year of vegetation

These results show that the repopulation of some natural habitats with *in vitro* obtained regenerants of *Rhodiola rosea* may be an alternative.

Conclusions

- The main morphogenetic reaction of shoot nodes and apices at *Rhodiola rosea* L. inoculated on several variants of MS medium was the formation of neoplantlets. The nutritive variants enriched with auxins improved the rhizogenesis;
- Callogenesis was a less intense process; it was obvious with the internode fragments when the medium variant was enriched with 2.4-D. Leaf and root parts provided an intense rhizogenesis and only sporadically shoots (in the presence of 2.4-D);
- We consider that the most efficient hormonal variants to micropropagate *R. rosea in vitro* from apices and shoot nodes are (in reversed order): N (2.0 mg l⁻¹ NAA), free hormone MS, KN (1 mg l⁻¹ Kin + 0.5 mg l⁻¹ NAA), AZ (0.2 mg l⁻¹ IAA + 2 mg l⁻¹ zeatin);
- *In vitro* regenerated neoplantlets were accommodated to septic environment in a hydroponic system in a short period of time, with no important losses of regenerants;
- The intensity of *in vitro* growth processes in *Rhodiola rosea* is much lower compared to its related species *Sedum hybridum* and *Sedum fabaria*.

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