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In vitro DIRECT SHOOT REGENERATION FROM *Rhodiola rosea* L. LEAF EXPLANTS

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Wild plant species are of great interest as a source of pharmacologically valuable compounds but a great number of them are endemic and/or endangered ones. Modern plant biotechnology can provide reliable methods for their utilization without disturbing natural populations. *In vitro* culture methods for *Rhodiola* species are being intensively developed to include them into various biotechnological programmes.

Aim. Development of a protocol for direct *Rhodiola rosea* L. plant regeneration from leaf explants.

Methods. The leaves of *R. rosea* aseptically growing plants were used as the explants. Several variants of Murashige and Skoog (1962) agar-solidified culture medium supplemented with different combinations of auxins (1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)) and cytokinins (kinetin and 6-benzylaminopurine (BAP)) were estimated as potential regeneration-inducing media. Regeneration frequency was calculated as the percentage of leaves that produced shoots.

Results. The use of MS medium supplemented with 2.5 mg/l BAP and 1.0 mg/l 2,4-D allowed inducing shoot formation with 100% frequency. An increase in the 2,4-D content up to 2.5 mg/l and decrease in BAP content to 1.0 mg/l resulted in decreasing of the regeneration frequency to 62.5%. Regeneration frequency was 25% and 62%, respectively, on the media containing 1.0 mg/l kinetin + 2.5 mg/l 2,4-D and 2.5 mg/l kinetin + 1.0 mg/l 2,4-D.

Conclusions. *R. rosea* leaf explants have demonstrated high regeneration capacity with the use of the studied combinations of plant growth regulators. MS medium supplemented with 2.5 mg/l BAP and 1.0 mg/l 2,4-D allowed inducing shoot regeneration in leaf explants with the frequency of 100%. The frequency of regeneration was lower in the case of substitution of BAP for kinetin. The other types of morphogenesis (formation of adventitious roots and/or callus) were also observed.

Key words: *Rhodiola rosea* L.; leaf explants; shoot regeneration; growth regulators.

According to different estimations the genus *Rhodiola* comprises from 130 to near 190 species of these almost 70 are accepted species names, and the others are synonyms or have not yet been clarified. *Rhodiola rosea* L. (synonyms *Sedum roseum* L. (Scop.); *Sedum rhodiola* DC.) also known as "golden root" or "roseroot" is dioecious, perennial plant of the family *Crassulaceae* distributed in mountainous regions of the Northern Hemisphere. It is included in the Red Lists of protected plant species in many countries [1–5].

For centuries, extracts of *R. rosea* roots were used in the traditional medicine as an adaptogen with various health-promoting effects to increase physical endurance, work productivity, resistance to high altitude sickness, and to treat fatigue, depression, anemia, infections, gastrointestinal ailments, and nervous system disorders. Modern phytotherapy considers it a vegetal source with an antioxidant and antistress-adaptogene action [1, 2, 5–8]. Biochemical studies of *R. rosea* rhizomes and roots have revealed the presence of six groups of compounds: phenylpropanoids (rosavin, rosin, rosarin), phenylethanol derivatives (salidroside, tyrosol), flavonoids, monoterpernes,

triterpenes, and phenolic acids. The pharmacological activity is based mainly on rosavin, rosin, and rosarin which are present in *R. rosea*, *R. sachalinensis*, *R. himalensis*, and *R. serrata* and salidroside found in the majority of *Rhodiola* species [4, 6, 7, 9]. It was shown that the content of these substances depends on the morphological part of the plant, its age and sex; the place and time of harvesting [4].

As *R. rosea* is an endangered medicinal plant its use from the natural habitats is restricted, and some new sustainable approaches are needed to avoid depletion of the natural sources. Field cultivation is challenging, costly and depending on climate and weather conditions; sufficient yields of roots/rhizomes could be obtained within 5–7 years. Chemical synthesis is another possible approach, and it has been already performed for rosavin and salidroside but not for the other biologically active compounds responsible for *Rhodiola* pharmacological properties [4, 9].

In vitro techniques provide controlled growing conditions, independency on the environmental factors, possibilities to optimize culture media, acceleration of biomass production, and ensuring of continuous production cycle [3, 5, 9]. To date, the *in vitro* cultures have been elaborated mainly for *R. rosea* and some Asian *Rhodiola* species, such as *R. crenulata*, *R. kirilowii*, *R. quadrifida* and *R. sachalinensis* [4, 5, 7]. Elaborated microclonal propagation methods can provide *in vitro* regenerated plants for repopulating native habitats of *Rhodiola* species as well as a raw material for secondary metabolites production [5, 7, 10, 11]. Shoots and roots cultured *in vitro* as well as callus and suspension cultures were studied as a source of biologically active compounds (salidroside, rosavin, triandrin, caffeic acid), and the impact of some stress factors, light and growth regulators on their production was estimated [1, 7, 12]. It was also shown that elicitation and biotransformation in *Rhodiola* cell cultures can be a feasible approach to sustainably enhance the content of active substances in *in vitro* cultures [1, 7, 12–15]. A promising way to enhance the secondary metabolite production by *R. rosea in vitro* cultures is the application of genetic engineering methods to regulate their biosynthetic pathways [5]. *Agrobacterium rhizogenes* was used to produce hairy roots of *R. rosea* [4, 9] and *R. kirilowii* [13] as a possible source of rosavinoids and salidroside.

The conditions of *R. rosea* shoot formation as a way for rapid multiplication were studied earlier [10, 11]. Plant growth regulators in different combinations were used. Leaves of aseptic plants are a fairly affordable type of explants to initiate growth of the new shoots. However, there is still lack of knowledge regarding the possibility of *R. rosea* direct shoot regeneration in leaf explants of aseptically growing plants.

The aim of the present research was elaboration of an efficient protocol for *R. rosea* regeneration in *in vitro* cultured leaf explants as a basis for including this species into further biotechnological studies.

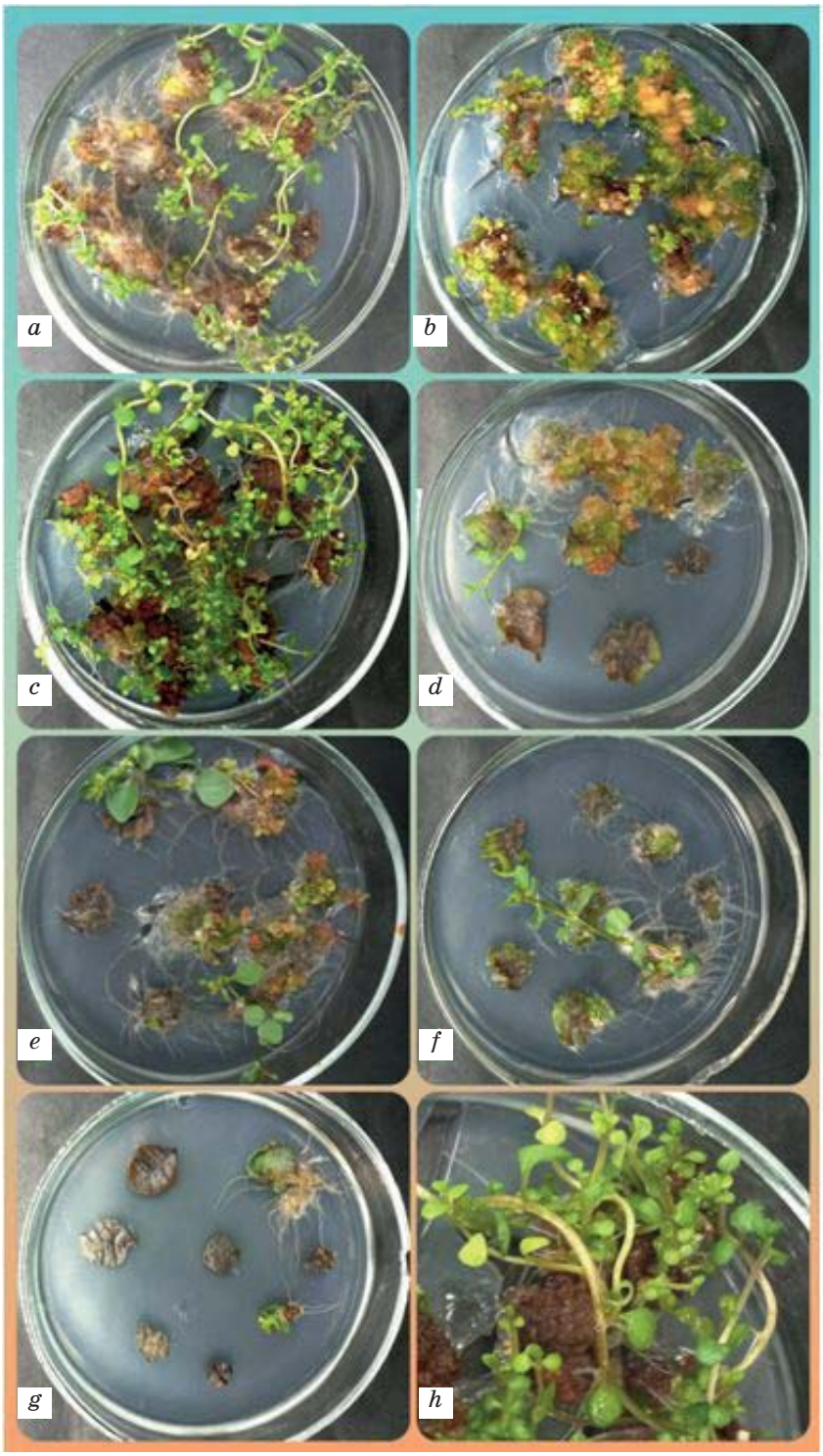
Rhodiola rosea L. plants from the collection of the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine were cultured *in vitro* on the solidified Murashige and Skoog medium (MS, Duchefa, the Netherlands). Leaves of aseptically growing plants were used as explants to study their regeneration capacities. The leaves with 3–5 incisions on them were cultured under 25 °C, 16-h/8-h photoperiod and the illumination of 3000 lux in Petri dishes on MS medium supplemented with 30 g/l sucrose and different combinations of the following plant growth regulators: 6-benzylaminopurine (BAP), kinetin, 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) (the Table). Combinations of plant growth regulators chosen for the experiments are the basic ones used for a number of plant species

***R. rosea* shoot regeneration on the different variants of culture media**

Medium, No	Growth regulators content, mg/l				Shoot regeneration frequency, %
	BAP	Kinetin	2,4-D	NAA	
1	1.0	–	–	0.5	87.5
2	1.0	–	2.5	–	62.5
3	2.5	–	1.0	–	100.0
4	–	1.0	2.5	–	25.0
5	–	2.5	1.0	–	62.0
6	–	1.0	–	0.5	37.5
7	–	–	–	–	0

held in *in vitro* collection of the Institute of Cell Biology and Genetic Engineering. *In vitro* morphogenesis (formation of shoots, roots and callus) was evaluated in the course of two months cultivation. The regeneration frequency was determined as the percentage of explants which formed shoots.

General results are shown in the Table. Hormone-free MS medium (No 7, the control variant) was not efficient for shoot production. The majority of explants did not form shoots, became necrotic and died, but sometimes root formation at their cut sites was observed (Fig., *g*).



Effect of growth regulators on shoot formation in *Rhodiola rosea* L. leaf explants:
media 1–7 (*a*–*g*); *h* — shoots formed on the MS medium with 2.5 mg/l BAP and 1,0 mg/l 2.4-D

On the medium supplemented with both 1.0 mg/l BAP and 0.5 mg/l NAA (medium No 1) direct regeneration of shoots was observes with 87.5% frequency during two weeks of cultivation. Besides shoot induction the mass root formation was detected with the frequency of 100% (Fig., *a*). Substitution of BAP for kinetin in the nutrient medium containing NAA (medium No 6, Fig., *f*) has led to the reduction of regeneration rate down to 37.5 %. Thus, with the same content of the auxin (NAA) in the media 1 and 6, replacing of BAP for kinetin reduced the ability of explants to form shoots.

Shoots were also induced on the medium No 2 but their growth started later, in three weeks, and the regeneration frequency was 625% (Fig., *b*). An increase in BAP concentration from 1.0 to 2.5 mg/l and decrease of 2,4-D content from 2.5 to 1.0 mg/l (medium No 3) significantly stimulated direct regeneration of shoots. Their formation was observed as early as in 10 days of cultivation with the regeneration frequency of 100% (Fig., *c, h*).

Substitution of BAP in the medium for another cytokinin, kinetin, in the presence of 2,4-D did not stimulate mass shoot regeneration in the leaf explants. Although shoot growth occurred under such conditions, the regeneration frequency was significantly lower than on the media supplemented with BAP. Thus, the regeneration frequency on the medium No 4 was no more than 25 %, and on the medium No 5 — 62 % (Fig., *d, e*). The attention must be payed to the almost identical regeneration frequency when media No 2 and No 5 with different growth regulators combination were used. Medium No 5 contained kinetin, and media No 2 — BAP. The latter regulator, as it turned out, sometimes has a more powerful effect as a phytohormone for regeneration. However, medium No 5 contained less effective kinetin, but in a much higher concentration.

A characteristic feature of morphogenesis on the medium No 4 is development of friable yellowish callus with interspersed pink areas. This may be an indirect indication of the synthesis of some secondary compounds inherent to *Rhodiola* plants. Root formation occurred in all media variants, but the most intense roots were formed on the culture media No 1 and No 5.

Root formation on the regenerated shoots was initiated after the shoots were transferred on hormone-free MS medium.

Cytokinins are usually used to induce multiple shoot formation so it is quite natural that the increase in cytokinin content contributed to the increase in regeneration frequency. At the same time, the fact is of interest that the regeneration of *R. rosea* shoots from leaf explants is also possible on the medium with auxin content of 2.5 times higher than that of the cytokinin.

Different combinations of plant growth regulators were used earlier for induction of *R. rosea* shoot formation and *in vitro* multiplication. However, in the majority of studies, the parts of plants with previously formed buds served as starting material. For example, shoot proliferation was demonstrated in pre-existing axillary buds on MS medium supplemented with thidiazuron or zeatin during 8 weeks cultivation [10]. Tasheva and Kosturkova [11] have studied apical buds, leaf nodes with leaves, stem segments, rhizome buds and segments to induce multiple shoots and have shown the differences in their regeneration rates. In our experiments, high regeneration capacity of the leaf explants of *R. rosea* aseptically cultured plants has been demonstrated, and the possibility of direct organogenesis has been proved. The elaborated regeneration protocols can be used both for *R. rosea* microclonal propagation and as a methodological basis for including this species into further biotechnological studies.

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ПРЯМА РЕГЕНЕРАЦІЯ ПАГОНІВ *in vitro* З ЛИСТКОВИХ ЕКСПЛАНТІВ *Rhodiola rosea* L.

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Дикорослі види рослин становлять великий інтерес як джерело фармакологічно цінних сполук, але багато з них є ендемічними та/або зникаючими. Сучасна біотехнологія може забезпечити надійні методи їх використання без порушення природних популяцій. Зокрема, інтенсивно розробляються методи культивування *in vitro* лікарських рослин роду *Rhodiola* для подальшого включення їх до різноманітних біотехнологічних програм.

Мета: розроблення протоколу прямої регенерації рослин *Rhodiola rosea* L. з листкових експлантів.

Методи. Використовували асептичні рослини з колекції Інституту клітинної біології та генетичної інженерії НАН України. Листки відокремлювали, робили на них надрізи та культивували на агаризованому живильному середовищі Мурасіге та Скуга (1962) з додаванням різних комбінацій таких регуляторів росту: 6-бензиламінопурин (БАП), кінетин, α-нафтилоцтова кислота (НОК) та 2,4-дихлорфеноксіоцтова кислота (2,4-Д). Оцінювали частоту регенерації як відсоток листків, на яких формувалися пагони.

Результати. Використання середовища з БАП (2,5 мг/л) та 2,4-Д (1,0 мг/л) дозволило індукувати пагони з найвищою частотою — 100%. Збільшення концентрації 2,4-Д до 2.5 мг/л та зменшення концентрації БАП до 1,0 мг/л привело до зниження показника частоти регенерації до 62,5%. На середовищах, що містили 1,0 мг/л кінетину + 2,5 мг/л 2.4-Д або 2,5 мг/л кінетину + 1,0 мг/л 2,4-Д частота регенерації становила відповідно 25% та 62%.

Висновки. Листкові експланти *R. rosea* показали високу регенераційну здатність на середовищах з різними комбінаціями регуляторів росту. Оптимальним середовищем для отримання регенерованих пагонів з листових експлантів з частотою 100% є середовище Мурасіге та Скуга з додаванням БАП та 2,4-Д у концентраціях 2,5 та 1,0 мг/л відповідно. Частота регенерації була нижчою при заміні БАП на кінетин. Спостерігали також інші варіанти морфогенезу (формування адвентивних коренів та/або калюсу).

Ключові слова: *Rhodiola rosea* L.; листкові експланти; регенерація пагонів; регулятори росту.