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Original Article

High Efficiency *in vitro* Plant Regeneration and Secondary Metabolite Quantification from Leaf Explants of *Rhodiola imbricata*

Ashwani Kumar Bhardwaj[1], Avilekh Naryal[1], Pushpender Bhardwaj[1], Ashish Rambhau Warghat[1,3], Balpreet Arora[2], Shikha Dhiman[1], Shweta Saxena[1], Pratap Kumar Pati[2], Om Prakash Chaurasia[1*]

Ashwani Kumar Bhardwaj[1], Avilekh Naryal[1], Pushpender Bhardwaj[1], Ashish Rambhau Warghat[1,3], Balpreet Arora[2], Shikha Dhiman[1], Shweta Saxena[1], Pratap Kumar Pati[2], Om Prakash Chaurasia[1*]

[1]Department of Biotechnology, Defence Institute of High Altitude Research, Defence Research and Development Organization, Leh-194101, INDIA.
[2]Department of Biotechnology, Guru Nank Dev University, Amritsar-143040, Punjab, INDIA.
[3]Department of Biotechnology, Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research, Palampur-176061, Himachal Pradesh, INDIA.

Correspondence

Dr Om Prakash Chaurasia

Scientist F & Director, Department of Biotechnology, Defence Institute of High Altitude Research, Defence Research and Development Organization, Leh-194101, INDIA.

Phone no : 0172-2638900

E-mail: opchaurasia2020@gmail.com

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ABSTRACT

Aim: *Rhodiola imbricata* is an endangered medicinal plant of the trans-Himalayan Leh-Ladakh region belonging to the family Crassulaceae. An efficient propagation and regeneration system via direct shoot organogenesis from leaf explant and evaluation of cinnamyl alcohol (Secondary metabolite) was established in this study. **Material and Methods:** *In vitro* grown leaves were inoculated using Murashige and Skoog (MS) medium supplemented with (alpha)-naphtalene acetic acid (NAA) in combination with 6- benzyladenine (BAP) for callus proliferation and regeneration. **Results:** The highest percentage of rhizogenous callus was induced in medium containing NAA (10.0-15.0 μM). The highest percentage of shoot formation from leaf derived callus was obtained in the medium containing of NAA (5.0 μM) and BAP (2.5 μM) as well as in NAA (1.0 μM), BAP (5.0 μM) (38.88% and 37.49%) respectively. Rooting of regenerated shoots were effective when a lower concentration of NAA (0.5 µM) was used alone. A maximum number of roots (22.0) and higher length (0.6 cm) was observed. The *in-vitro* plantlets with well-developed shoots and roots were acclimatized successfully to natural field conditions with a survival rate of over 80%. Cinnamyl alcohol (Secondary metabolite) evaluation was also done for the very first time and an upregulation of 49.6% and 30.6% were observed in *in-vitro* roots and shoots when compared with mother root and shoot respectively. Hence, it was proved that the content of secondary metabolites obtained from *in-vitro* raised plants is higher than mother plant. **Conclusion :** These results may lay a foundation for genetic improvement and can be used to determine sequential level of targeted secondary metabolites through cell culture in *Rhodiola imbricata*.

Key words: Regeneration system, Acclimatization, Endangered, Plant growth regulator, Cinnamyl alcohol.

INTRODUCTION

The trans-Himalayan cold desert is located to the north of the great Himalaya that includes Karakoram, Ladakh, Zanskar and Kailash mountain ranges.[1] The trans-Himalayan cold desert region of India is a repository for an abundance of medicinal plants.[2] Medicinal value of these plants can have a direct benefit for human health and can be helpful in promoting various therapeutic formulations.[3] High altitudinal medicinal plants survive in extreme conditions such as low temperature, high Ultraviolet intensity and less oxygen.[4] This stress condition helps in increasing secondary metabolite production.[5] These plants have been used for medico-religious purposes which in turn help in generating income for local people.[6] Out of several medicinal plants found in trans-Himalayas, *Rhodiola imbricata* frames to be a distinctive herb due to the presence of various phytopharmaceutical constituents which can be helpful for human kind.[7]

Rhodiola imbricata is a worldwide popular plant with valuable usages as medicine which is found in high altitude area of Asia and Europe.[8,9] *Rhodiola* species is a member of the Crassulaceae family and it is also known by different names such as Himalayan stone crop, Himalayan rose root and Golden root. This grows on rocky slopes, common in drier areas of the western Himalayan at an altitude of 4000-5000 m.[8,9,10] *Rhodiola* species found at high-altitude passes of Northern Hemisphere. It is a succulent herb with a thick rhizome, golden outside, pink inside, 10-35cm with rose scented massive rootstock; leaves 13.3 cm long, oblong to narrow elliptic. Morphologically, it has two series of stamens, twice the number of petals, and free petals (Flora of China 2001). Flowering and fruiting occur in July-September.[8,11] This plant is dioecious and rarely hermaphroditic.[12,13]

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This herb is succulent and locally known as rose root due to rose like attar (fragrance) of fresh cut rootstock.[8]

This medicinal herb biosynthesizes phytochemicals such as flavonoids, coumarins, phenolic acids, Phenylethanoids, phenylpropanoids.[14] Plant root extracts possesses pharmacological activities. In some countries, *Rhodiola* extract has been used in anti-stress and anti-fatigue drinks.[15] It is well documented that *R. imbricata* rhizome stimulates the innate immune pathway and has potent immune stimulatory activity and hence it is also used in modulating the immune system of immune compromised individuals.[16] The roots of *Rhodiola imbricata* are used against lung problems, cold, cough, fever, loss of energy and pulmonary complaints.[17,18,10] Among all of them, Salidroside, Rosin, and its derivatives are mainly used in a medicinal system.[13] Salidroside is initially reported from *Rhodiola* plants in the 1970s.[13,19] and further studies stated that salidroside but also rosin derivatives are important bioactive compounds.[13,20,21,22] These compounds have bioactivities like adaptogenic which decreased cellular sensitivity of stress.[23] Salidroside is a radioprotective and it considerably reversed senescence or anti-aging.[24] radioprotective[13,9] In recent years, this plant has gained considerable interest because of its physiochemical properties.

Biotechnological methods are frequently used to generate a large number of consistent plant material under controlled conditions independent of environmental stresses like biotic and abiotic.[13] Development of an efficient regeneration system is a pre-requisite for its manipulation and improves the economic and commercial value of *R. imbricata*. Therefore, the present study was designed to develop an efficient protocol for direct shoot regeneration from leaf explants of the *Rhodiola imbricata*, which possesses higher concentrations of cinnamyl alcohol (Secondary metabolite). This system will be further useful for the mass production of secondary metabolites from this plant for commercial purpose.

MATERIAL AND METHODS

Source and explants sterilization

For regeneration experiments, young expanded leaves of *R. imbricata* were collected from tissue culture laboratory, Defence institute of high altitude research, Leh-Ladakh. Leaf surface sterilization was done with autoclaved distilled water thrice. Mercuric chloride (Merck) 0.04% (w/v) having one drop of TWEEN 20 for 2–3min was added and was thoroughly washed with autoclaved distilled water 4–5 times to remove the remaining traces of sterilizing agents.[25]

Callus proliferation and shoot regeneration

Leaves were cut into small pieces and inoculated in culture tubes, containing MS medium supplemented with 3% sucrose and 0.8% agar fortified with various combination of 1-Naphthaleneacetic acid NAA (0, 0.5, 1.0, 2.5, 5.0 μM) and Benzyl amino purine (BAP) (0, 0.5, 1, 2.5, 5, 10μM). Inoculated cultures were incubated under a cool white fluorescent light in the culture room. After four weeks of inoculation, callus induction and regeneration response were recorded.

Rooting of Microshoots

For rooting of *in vitro* raised shoots, different concentrations of NAA (0, 0.5, 1.0, 2.5, 5 μM), IAA (0, 2.5, 5, 10 μM), IBA (0, 2.5, 5, 10 μM) were used. Cultures were incubated at 25±2°C with 55±5% relative humidity in the culture room under a cool-white fluorescent light. The rooting response of shoots was recorded after four weeks.

In vitro hardening and acclimatization to natural field

Four weeks of rooted micro shoots were used for hardening; the rooted shoots were transferred to pots containing ratio of 1:1:1 soil, perlite and vermiculite. The plants were initially covered with poly bags and kept in a culture room at 25±2°C with 55±5% relative humidity under a cool white fluorescent light. After seven days, polybags were removed, and plants were directly exposed to light. All experiments were conducted thrice with 30 replicates for shoot regeneration and rooting.

RP-HPLC analysis of secondary metabolite Plant Extraction and RP-HPLC analysis

Plant material (roots and shoots) was collected from the *in-vitro* raised plant as well as from *ex-situ* population. Collected samples were air dried and grounded to a fine powder using pestle, mortar. Powdered samples were defatted with 10 ml of 100 % methanol and were sonicated using the ultrasonic bath for 15 min each. Procedure has been repeated three times for better outcome. All the three aliquots were pooled to 15 ml and vacuum-dried with the help of a rotavapour (Buchi, Switzerland). Dried residues were reconstituted with 5 ml methanol and filtered with a 0.22-μM syringe filter (Milex GV; 13 mm). The extracts obtained were used for qualitative and quantitative analysis by Reverse phase–High-performance liquid chromatography method. Briefly, RP-HPLC analysis were performed on Agilent 1260 Infinity series HPLC (Agilent Technologies, Santa Clara, CA) equipped with Quaternary Pump VL (G1311C) and degasser, 1260 ALS auto-sampler (G1329B) and 1260 DAD VL detector (G1315D). Separations were achieved using ZORBAX Eclipse C18 column (4 X 100 mm) (Agilent Technologies). The mobile phase consisted of 25mM phosphate buffer (A), adjusted to pH 7.0 and acetonitrile (B), gradient elution (95A/5B) for 30 min to (80A/20B). 5 min washing was given with 0.1% phosphoric acid in methanol maintaining an equilibration period of 7-10 min. The flow rate was adjusted to 1.0ml/min and wavelength detection was set to 255 nm with 5ml of sample injection. Separation was done at 60°c. Peaks were assigned by spiking the samples with standard compounds, and were compared with UV-spectra and retention time. The results were expressed as mg/g DW of the sample.

Statistical data analysis

Sample data were analyzed using one-way analysis of variance (ANOVA) and are presented as Means ± Standard error using Duncan multiple range test at the 5% probability level. The data were analyzed using IBM SPSS for window version 23(SPSS Inc., Chicago, IL, USA).

RESULTS

Callus proliferation

Callus induction was started after 3-4-week cultures on NAA and BAP supplemented media (Table 1). It was observed that at higher concentration of NAA (10.0-15.0 μM) produce rhizogenous callus and lower concentration of NAA (2.5 μM) produce very less quantity of green compact callus. Further, low concentrations of BAP (0.5-1.0 μM) leads to direct regeneration from leaf explants and higher (2.5-10 μM) concentration lead to callus formation. The better response of explants for callus formation (green compact callus 100%) was observed in the MS medium supplemented with NAA (10.0 μM) with BAP (5.0 μM) and rhizogenous callus was obtained in NAA (10.0 μM) and BAP (0.5 μM) concentrations respectively.

Effects of PGR concentration and combination on shoot proliferation from *in-vitro* leaf explants

Direct shoot regeneration (11.11%) was started from leaf explants, media containing NAA (0.5 μM) and BAP (1.0 μM). Multiple shoot regeneration (38.88% to 37.49%) was more pronounced by the sub-culturing of callus on to the MS medium supplemented with NAA (5.0 μM) and BAP (2.5 μM) as well as in NAA (1.0 μM) and BAP (5.0 μM), respectively (Figure1).

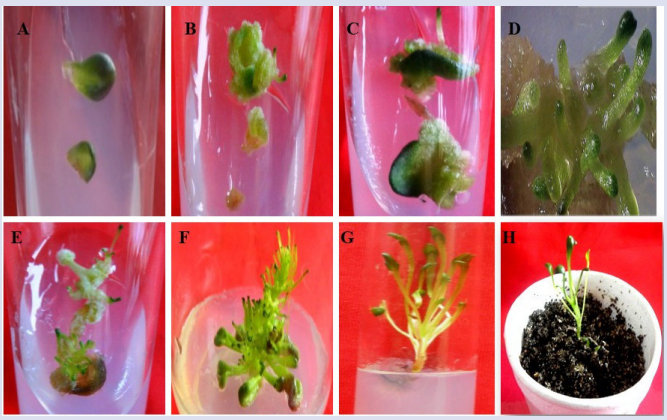

Figure 1: Schematic representation of shoot regeneration in *Rhodiola imbricata* (A) leaf explant (B) Initiation of callus induction from explant in ms medium having NAA+ BAP (C) Proliferation of callus after 14 days, (D) Shoot bud formation after 20 days (E) microshoots transferred in MS medium supplemented with BAP (F) Shoot proliferation in MS medium supplemented with BAP (G) *In vitro* rooting of microshoots in agar gelled medium supplemented with NAA, (H) hardening of *in vitro* rooted plants.

Figure 2: Schematic representation of in vitro root induction in *Rhodiola imbricata* (A) *In vitro* rooted shoots in agar gelled medium supplemented with auxin, (B) Hardening of *in vitro* rooted plants.

In vitro rooting

Effect of different concentrations and types of auxin on root induction was observed (Table 2). From present data, it was observed that type and concentration of auxins influenced the number and length of roots per explants. Auxins such as NAA, IBA and IAA were used, to optimize the effective auxin concentration for root induction. MS medium supplemented with different auxin significantly increased the rate of root multiplication. It was observed that NAA (0.5 µM) showed the highest number (22.0±1.1) of roots followed by 2.5µM of IBA (20.33 ± 6.11). It was also observed that in case of IBA and IAA gradual increase in auxin concentration resulted in a decrease in root number. The increasing root length was observed at a lower concentration of NAA (0.5µM) followed by IBA (2.5 µM) (0.6cm). In contrast, it was also observed that increase in IBA and NAA concentration resulted in a decrease in root length. All the three hormones were tested but NAA showed high root induction followed by IBA and IAA being the least.

Table 1: Effect of different plant growth regulators on regeneration and callus induction.

NAA	BAP	Callus induction percentage	Regeneration in percentage
0	0	0.00±0.00^{f}	0.00±0.00^{f}
0.5	0	0.00±0.00^{f}	0.00±0.00^{f}
1	0	0.00±0.00^{f}	0.00±0.00^{f}
2.5	0	44.44±1.11^{abcd}	0.00±0.00^{f}
5	0	49.99±5.55^{abcd}	0.00±0.00^{f}
10	0	61.16±5.50^{abc}	0.00±0.00^{f}
0.5	0.5	0.00±0.00^{f}	22.22±11.11^{ab}
1	1	76.78±1.78^{abc}	8.33±0.00^{c}
2.5	2.5	88.88±11.11^{a}	0.00±0.00^{f}
5	5	94.44±5.56^{a}	0.00±0.00^{f}
10	10	100±0.00^{a}	0.00±0.00^{f}
0.5	0.5	0.00±0.00^{f}	0.00±0.00^{f}
1	0.5	0.00±0.00^{f}	0.00±0.00^{f}
2.5	0.5	83.32±5.55^{ab}	0.00±0.00^{f}
5	0.5	83.32±5.55^{ab}	0.00±0.00^{f}
10	0.5	80.80±8.08^{ab}	0.00±0.00^{f}
0.5	1	16.66±5.55^{abcde}	11.11±0.00^{ab}
1	1	90.00±10.00^{a}	9.72±1.39^{ab}
2.5	1	100.00±0.00^{a}	0.00±0.00^{f}
5	1	90.00±0.00^{a}	0.00±0.00^{f}
10	1	100.00±0.00^{a}	0.00±0.00^{f}
0.5	2.5	66.66±0.00^{abc}	0.00±0.00^{f}
1	2.5	61.10±5.55^{abc}	39.33±0.00^{a}
2.5	2.5	100.00±0.00^{a}	23.61±1.39^{ab}
5	2.5	72.21±5.5^{abc}	38.88±5.5^{a}
10	2.5	95.00±5.00^{a}	0.00±0.00^{f}
0.5	5	72.21±5.5^{abc}	0.00±0.00^{f}
1	5	62.49±4.16^{abc}	37.49±4.16^{a}
2.5	5	94.44±5.56^{a}	11.11±0.00^{ab}
5	5	95.83±4.17^{a}	12.49±4.16^{ab}
10	5	100.00±0.00^{a}	29.16±4.16^{a}
0.5	10	11.72±0.50^{abcde}	0.00±0.00^{f}
1	10	22.22±0.00^{abcde}	0.00±0.00^{f}
2.5	10	83.88±5.5^{abcde}	0.00±0.00^{f}
5	10	83.33±0.00^{ab}	0.00±0.00^{f}
10	10	83.33±0.00^{ab}	0.00±0.00^{f}

Data recorded after 4 weeks of inoculation in culture tubes; results are represented as Mean ± SE, each concentration consisted of 3 replicates. Same letters within a column are not significantly different (Duncan's multiple range test, P<0.05).

Hardening and acclimatization of *in-vitro* raised plantlets

In vitro raised plantlets with well-developed shoots and roots were transferred to pots containing soil: perlite: vermiculite (1:1:1) and were kept

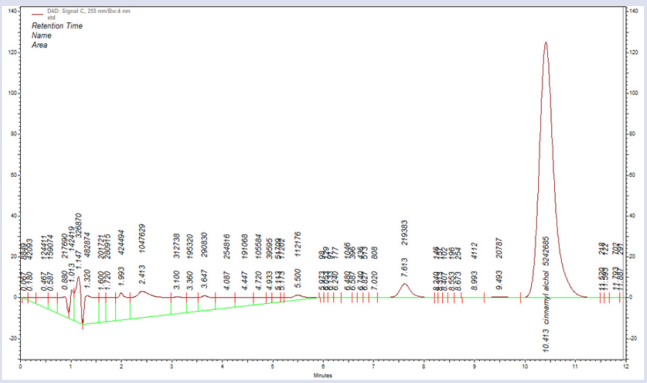

Figure 3: Reference standard Targeted secondary metabolite (Cinnamyl alchol) using high-performance liquid chromatography.

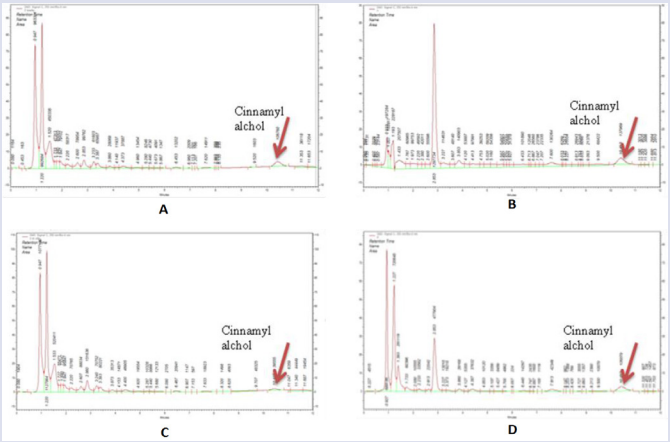

Figure 4: Analysis of Targeted secondary metabolite (Cinnamyl alchol) from *Rhodiola imbricata in-vitro* vis-à-vis mother plant. A- *In-vitro* Root, B- Mother Root, C- *In-vitro* Shoot, D- Mother Shoot using high-performance liquid chromatography.

Data recorded after 4 weeks of inoculation in culture tubes; results are represented as Mean ± SE, each concentration consisted of 3 replicates. Same letters within a column are not significantly different (Duncan's multiple range test, P<0.05)

in a hardening chamber at 25 ± 2 °C with 55±5% relative humidity under white fluorescent light. After four weeks of transfer, the successfully acclimatized plants (80%) were transferred to pots under sunny condition where they grew well without any detectable phenotypic variation.

Secondary metabolite content detection

Metabolite analysis of *in vitro* raised plants was done using RP-HPLC. Among different metabolites reported in the literature we focused on Cinnamyl alcohol. As a result a very interesting observation was observed. cinnamyl alcohol was found to be significantly increased in tissue culture raised plants as compared to naturally grown plants. 120 days of *in-vitro* raised shoots showed 30.6 % up-regulation in cinnamyl alcohol when compared with mother shoots whereas *in-vitro* raised roots showed a significant increase of 49.6% compared to mother root. (Table 3)

Table 2: Effect of different concentration of auxins on root induction in *R. imbricate*.

Concentration of growth regulator (µM)	Root number	Root length (cm)
0.0	4.00±0.577[a]	0.167±0.033[a]
IAA		
2.5	9.667±1.764[b]	0.367±0.0882[a]
5	11.333±2.028[b]	0.500±0.115[ab]
10	8.0±0.577[c]	0.323±0.0882[ab]
IBA		
2.5	20.33±6.119[b]	0.60±0.0667[b]
5	17.00±0.577[b]	0.33±0.066[a]
10	10.00±1.732[a]	0.33±0.0882[a]
NAA		
0.5	22.000±1.155[b]	0.633±0.033[b]
1	15.667±1.202[bc]	0.433±0.033[c]
2.5	12.333±0.882[c]	0.267±0.033[a]
5	10.667±0.667[a]	0.200±0.0577[a]

Data recorded after 4 weeks of inoculation in culture tubes; results are represented as Mean ± SE, each concentration consisted of 3 replicates. Same letters within a column are not significantly different (Duncan's multiple range test, P<0.05)

DISCUSSION

In vitro regeneration is successfully used for rapid multiplication of plant material and conservation of elite varieties. It is well known that, a complete *in vitro* regeneration protocol needs to be established for each explant of each species, and for each culture stage, in terms of nutrient medium, temperature, length, age and importantly, endogenous level of phytohormones. In medicinal plants, it can be used as a model system to understand the biosynthetic pathways of their pharmaceutically important secondary metabolites. It can also be used for modulating these biosynthetic pathways to enhance biosynthesis of secondary metabolites. Various explants such as seeds, leaves, stamen, roots and meristems etc. have been used to initiate the aseptic cultures of *Rhodiola imbricata* for rapid multiplication of plant material and production of secondary metabolites. Apical buds and stem nodes from *in vitro* seedlings were also used for micro propagation and regeneration studies in other species of *Rhodiola*.[26,27] The present research finding, reports first ever study of *in vitro* adventitious shoot regeneration from leaf explant of *Rhodiola imbricata*.

In vitro shoot, regeneration and callus induction either directly from explants or indirectly from callus have many therapeutic and commercial applications such as large scale propagation of superior genotypes and improvements in genotype.[28] In regeneration studies of *R. imbricata*, leaf explants were used for initiation of callus. Callus proliferation has been done using optimum cytokinins-auxins concentrations and has been reported in other plants.[29] The concentration of plant growth regulators appears to be the most important factor for high frequency shoot regeneration. Leaf explants responded well to both growth variants (NAA and BAP). Higher concentration of BAP produced better callusing response. The highest callus (100%) response from the leaf explant was observed at NAA (2.5 µM) and BAP (2.5 µM). Different concentration of NAA and BAP were used alone as well as in combination for prolific shoot regeneration studies. Shoot regeneration frequency showed the clear effects of concentration of growth variants. It was observed that BAP and NAA when used alone did not show any significant regeneration.

Table 3: Quantification of targeted secondary metabolite (cinnamyl alchol) using Reverse Phase high-performance liquid chromatography.

Secondary Metabolites (mg/g DW)	IVR	IVS	MR	MS
Cinnamyl alchol	0.0181±0.0635[a]	0.0128±0.532[b]	0.0121±0.0587[c]	0.0098±0.265[d]

Results are represented as Mean ± SE, each concentration consisted of 3 replicates. Same letters within a column are not significantly different (Duncan's multiple range test, P<0.05)

NAA alone did not induce any regeneration response at lower concentration (0-2.5 µM). Similarly, irrespective of any concentration (0-15 µM) BAP did not show any regeneration response. However, a better regeneration response was obtained when lower concentration of NAA (0.1 µM) along with a different concentration of BAP were used. The highest regeneration response (38.88%) was observed at NAA (5 µM) and BAP (2.5 µM). This may be attributed that higher concentrations of cytokinin and auxin, higher will be the regeneration response. Similar observation was reported in *Begonia homonyma*.[30] This study implicated that for shoot regeneration, higher concentration of PGR's are needed, whereas for rooting lower concentration of these hormones work.

Further, for optimization of root induction three auxins such as NAA, IBA and IAA with different concentration were used. NAA (0.5 µM) found to be more effective for rooting. An increased number of roots (22.0) and higher length (0.6 cm) were observed at a lower concentration of NAA whereas an increasing concentration of NAA causes reduced in root number and length. The rooting efficiency and biomass concentration were significantly increased in NAA (0.1 µM) compared to other concentrations. Also, higher concentration of NAA was observed to be best for induction of rhizogenous callus. However, IAA did not show any significant effect on rooting at a lower concentration. However, in case of IAA, an increasing trend of root number was observed from 2.5 µM to 10 µM. Further, gradual increase of IBA concentration resulted in a decrease in root number. In case of root length, different auxin responded differently. The increasing root length was observed at a lower concentration of NAA (0.5µM) followed by IBA (2.5 µM) which showed 0.6 cm and increase in IBA and NAA concentration resulted in a decrease in root length. Similar effect has been studied by other groups in other plant species.[31]

Efficient rooting of *in vitro* regenerated plants and subsequent field establishment is the last and crucial stage of rapid clonal propagation. *In vitro* raised plantlets with well-developed shoots and roots were transferred to pots containing sterile soil, perlite and vermiculite, after which the successfully acclimatized plants (80%) were transferred to pots without any detectable phenotypic variation (Figure 2). Similar results were achieved by Ishmuratova *et al*., 1998 in other species of *Rhodiola*.

Further HPLC estimation of secondary metabolite was done and interesting observation was noticed for the very first time. An upregulation of nearly 46% and 30 % were observed in roots and shoots of *in-vitro* regenerated plants as compared with mother plants respectively. This increase in secondary metabolite accumulation in *in vitro* raised plants is an indication that growth hormone application has influence on this pathway. Modulation of secondary metabolites production by plant growth regulators is very old.[32] Similar comparative studies were reported for *Solidago chilensis, Solanum villosum* and *Exacum bicolor*.[33,34,35] The present study showed that the choice of hormones type and concentration exogenously supplied during tissue culture markedly influences not only shoot proliferation but also the *in vitro* production of bioactive secondary metabolites.

CONCLUSION

This is the first ever study for the successful callus proliferation, direct regeneration from leaf explants and regeneration through callus in *R. imbricata*. Thus, results achieved a clear, simple and reliable protocol for large scale multiplication. The research findings showed that tissue culture techniques can play an important role in clonal propagation of elite genotypes which has diverse medicinal applications and eventually due to over exploitation and irregular concern this plant is facing local extinction. In view of the medicinal properties and increased demand of this plant in the pharmaceutical industry, the outlined procedure offers a potential system for improvement and conservation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS USED

IVR: *In-vitro* Roots; **IVS:** *In-vitro* Shoots; **MR:** Mother Roots; **MS:** Mother Shoots; **IAA:** Indole Acetic acid.

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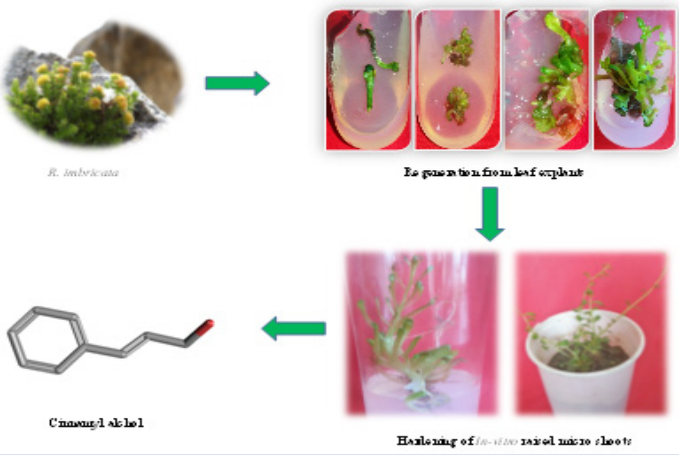
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Ashwani kumar Bhardwaj: Is Senior Research Fellow and Ph.D scholar in the Plant Science Division, DIHAR, Leh. He currently works on a project entitled 'Conservation and propagation of Rhodiola imbricats – a high value medicinal plant of trans Himalayan Leh Ladakh region. He holds M.Sc in Biotechnology from the Shoolini University Himachal Pradesh.

Om Prakash Chaurasia: Is Scientist 'F' and Director, Defence Institute of High Altitude Research. He obtained his Ph.D degree in Botany from Magadh University Bodh Gaya, Bihar in 1992. He has extensively surveyed trans-Himalayan belts of Ladakh and Lahaul-Spiti and documented the fragile plant biodiversity and its ethnobotanical wealth. Two Research Fellows have been awarded Ph.D. under his supervision.

Avilekh Naryal: Is Senior Research Fellow and Ph.D scholar in the Plant Science Division, DIHAR, Leh.

Pushpender Bhardwaj: Is Senior Research Fellow and Ph.D scholar in the Plant Science Division, DIHAR, Leh.

Ashish Rambhau Warghat: Is a Scientist in CSIR IHBT palampur and currently working on conservation of medicinal plants through plant tissue culture.

Balpreet Arora: Is a project trainee in department of biotechnology ,Guru nank dev university, Amritsar.

Shikha Dhiman: Is a project trainee in department of biotechnology DIHAR,DRDO , Leh Ladakh.

Shweta Saxena: Is Scientist 'E' and Head, Plant Science Division at DIHAR, base Laboratory, Chandigarh.

Pratap Kumar Pati: Is a professor in Guru nank Dev university Amritsar.

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