ELICITATION OF SALIDROSIDE UNDER TISSUE CULTURE CONDITIONS IN THE TRANS-HIMALAYAN PLANT *RHODIOLA IMBRICATA*

Project Report submitted in partial fulfillment of the requirement for the degree of

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IN

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By

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UNDER THE GUIDANCE OF

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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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DECLARATION

We hereby declare that the report entitled**, "Elicitation of Salidroside under tissue culture conditions in the Trans Himalayan plant *Rhodiola imbricata*"** has been solely submitted at **Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat** in partial fulfillment for the award of degree of B.Tech in Biotechnology. The work has not been submitted elsewhere for any other degree, diploma or appreciation.

The concept and hypothesis behind the research work and experiments were planned and executed under the guidance of our supervisor**, Dr. Hemant Sood** and hence, it's a work of complete originality. Wherever any experimental data or materials (data, analysis, figures, tables, texts, graphs) from other sources has been used, we have given due credit by citing them in text of the thesis.

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CERTIFICATE FROM SUPERVISOR

This is to certify that the work embodied in the accompanying project report entitled **"Elicitation of Salidroside under tissue culture conditions in the Trans Himalayan plant *Rhodiola* imbricata",** pursued by **Anaida Kad** and **Archit Pundir** in partial fulfillment for the award of degree of B.Tech in Biotechnology from **Jaypee University of Information Technology, Waknaghat** has been carried out under my supervision. This work has not been submitted partially or wholly to any another University or Institute for the award of any other degree, diploma or appreciation.

Signature of Supervisor -

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In gratitude,

Anaida Kad

Archit Pundir

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List of Abbreviations

ABBREVIATIONS	FULL FORM
HPLC	High Performance Liquid Chromatography
MS	Murashige and Skoog
IBA	Indole-3-butyric acid
KN	Kinetin
BAP	6-Benzylaminopurine
IL-2	Interleukin-2
ROS	Reactive oxygen Species
H_2O_2	Hydrogen peroxide
KLH	keyhole limpet hemocyanin
IgG	Immunoglobulin G
CD	Cluster of Differentition
TNF-α	Tumor necrosis factor-α
NF-κβ	Nuclear factor-κβ
UVB	Ultraviolet B
IL-6	Interleukin 6
DOPA	Dihydroxyphenylalanine
PBMC	Peripheral blood mononuclear cell
PAL	Phenylalanine ammonialyase
Phe	Phenylalanine
Tyr	Tyrosine
TyrDC	Tyrosine decarboxylase
RcTyrDC	Recombinant Tyrosine decarboxylase
RAE	*Rhodiola* aqueous extract
NK	Natural killer
CCR	Cinnamyl-CoA reductase
CAD	Cinnamyl alcohol dehydrogenase
DRDO	Defence Research and Development Organisation
CCR	Cinnamyl-CoA reductase
PARP1	Poly (ADP-Ribose) Polymerase 1
TGase	Transglutaminase
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase
PeGs	phenylethanoid glycosides
GA_3	Gibberellic acid
TDZ	Thidiazuron
Tert-BHP	Tert-butyl hydroperoxide
HCl	Hydrochloric acid
NaOH	Sodium hydroxide

ABSTRACT

The herbaceous, *Rhodiola imbricata* belonging to Crassulaceae family, is a dioecious perennial plant. It is indigenous to the Trans- Himalayan region and exclusively found in Leh-Ladakh valleys of India and border regions of China and Tibet. Various pharmacological activities in *R. imbricata viz.* hepatoprotective, radioprotective, immunomodulatory etc., are attributed to presence of different phytochemicals such as p- tyrosol, salidroside, rosavin and rosin. But the established plant cell cultures of *Rhodiola imbricata* usually encounter problems of low product yields and high cost, which discourage its commercialization. Moreover, the quantitative analysis and HPLC studies of these metabolites in *R. imbricata* proved their concentration to be relatively less in comparison to other species. So, in order to enhance the secondary metabolite content in *R. imbricata,* we have studied the effect of different abiotic elicitors on marker compound production. Owing to the previous success of different chemical elicitors on other species of *Rhodiola,* we have performed elicitations on the callus as well as shoot cultures of *Rhodiola imbricata.* Along with this, for the first time we have studied the effect of photosynthetic growth regulator light, ultraviolet light, ultrasonic waves and electric shock on the biosynthesis and accumulation of salidroside. The best shoot growth in elicited cultures was reported under Photosynthetic light and data were collected for number of shoots 8±0.33 with average shoot size 6±0.63 cm, number of leaves 31±0.16, leaf size 1±0.28 cm and biomass 10±0.96 g. The highest salidroside content 3.117±0.008 mg/g was obtained by using UV light as an elicitor.

Liquid MS media supplemented with different growth hormones have been tried for improving the biomass of *R. imbricata* under in vitro conditions. The best results were obtained in liquid MS medium supplemented with BAP (1 mg/L) + IBA (2 mg/L) with 87±0.95 g biomass yield in 3 weeks duration of time. The relative enhancement achieved in liquid media in comparison to solid media is 5.35 folds more.

So, the current study develops a scope for future explorations in marker compound production at commercial scale.

CHAPTER 1

INTRODUCTION

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INTRODUCTION

Rhodiola imbricta Edgew. is a herbaceous, dioecious perennial plant, belonging to Crassulaceae family. There are nearly 1400 species in stone crop family which are distributed in 33 genera with *Rhodiola* present worldwide especially in the regions of South Africa and Northern Hemisphere [1].

The *Rhodiola* spp. consists of about 130 species, [2] and many *Rhodiola* species have been used traditionally for curing chronic illness and weakness in the regions of Tibet and Western Himalayas belt for over 1000 years [3]. In India, there are six species of *Rhodiola,* which are, *R .tibetica, R. imbricata, R. quadrifida, R. sinuate, R. heterodont, and R. wallichiana* [4].

The common names of *Rhodiola imbricata* are Rose root (due to the rose- like fragrance of the fresh cut rootstock), Golden root, Arctic root, Shrolo (as commonly called by the locals of Ladakh region), Solo (by localites of Rohtang- Manali region) and stone crop or Himalayan stone crop in India because it grows along the stones only [5].

1.1 Geographical Distribution

R. imbricata Edgew was originally thought to have originated in the Himalayas and mountainous regions of southwestern China, today it is distributed not only in mountainous but also coastal habitats [6]. The plant is native to the entire northern hemisphere [7]. In India, *R. imbricata* grows in the Trans-Himalayan cold desert, high arctic latitudes and mountainous regions of Eurasia, mainly on rocky slopes, wetlands and higher elevation passages (14000-18500 ft. It is commonly found in the Indus Valley and Leh of India Trans-Himalaya [5, 7].

According to the Indian biodiversity portal, this plant has been seen in three places: the Rohtang Pass, Himachal Pradesh; Khardung La Pass, Jammu and Kashmir; Birje Ganj pass, Uttarakhand.

1.2 Morphology

It is an erect, succulent herb which reaches up to the height of 10-35 cm, with a thick rhizome, golden outside, pink inside.

Leaves: Leaves are generally 1.3-3 cm long, oblong to narrow elliptical and nearly entire.

Roots: The plant has a massive rose scented rootstock.

Flowers: Flowers are pale yellow in colour in the form of congested clusters, surrounded by an involucres of leaves.

Fruits: Fruits are generally 4-5 and have many seeds.

The general flowering and fruiting season occurs in July-September [5, 7].

1.3 Taxonomy

In third world countries or underdeveloped countries, about 80% of people depend mainly on traditional medicine for health care needs. An important part of the health sector includes the use of plant extracts or bioactive compounds formed by plants for general treatments. Quality control and standardization profiles for accurate recognition of the species, whether fresh, dry or powdered, are one of the basic requirements of medicinal plants [9]. In the preparation and administration of medicinal herbs, the real threat is faulty change and misclassification of species [9]. Most herbs that are confused with each other are herbs that seem extremely comparable to the untrained eye. The classification of defective species and the erroneous substitution of plants also led to serious adverse effects [9]. Therefore, the taxonomic and botanical classification and the recognition of specific species of their natural habitat of *R. imbricata* mentioned previously in the introduction are very useful..

The current taxonomic status of the *Rhodiola* genus is complex due to the generally similar morphology [6, 10]. According to GBIF (2010), the genus *Rhodiola* includes 136 accepted species, while the list of plants (http://www.theplantlist.org) includes 135 species of scientific plants from the range of species for the genus *Rhodiola*. Of these 61 names of accepted species, 16 have not yet been clarified.

1.4 Chemical Composition

Analysis of different *Rhodiola* species revealed six groups of active principles in their chemical compositions [8].

- **Phenylpropanoids**: The name Rosavin includes these three: Rosin, Rosavin, Rosarin.
- **Flavonoids**: Acetylrhodalgin, Rhodionin, Rhodiosin, Rhodiolin, Tricin.
- **Phenylethanol derivatives**: Tyrosol, Rhodioloside which includes: Salidroside and Rhodosin.
- **Triterpenes**: β-sitosterol, Daucosterol.
- **Phenol acids**: Hydroxycinnamic, Chlorogenic and Gallic acids.
- **Monoterpenes**: Rosiridol.
- The presence of Triandrine, p-coumaric alcohol and its glucosides (Vimalin), p-cumaric acid, Caffeic acid, β-sitosterol, Daukosterol and Salidroside (in trace amounts) has also been detected in callus tissues cultures [9].

  Initially, in the 1970s, the compound responsible for unique pharmacological properties of *Rhodiola* genus was believed to be Salidroside [12]. According to the Russian Pharmacopeia (1989), the raw material of *R. rosea* should contain 0.8 % salidroside [11]. However, further studies revealed that not only salidroside but also rosin derivatives are important bioactive compounds [12 - 14].

1.5 Indigenous Uses

In Tibet, Mongolia and other regions, Rhodiola species have been used as traditional medicines for more than 1000 years for the treatment of eternal weakness and diseases caused by infections [3]. *R. imbricata* is not only an important traditional medicinal plant, but it is also widely used as a food crop and distributed in the cold regions of the trans-Himalayan desert. The roots of *R. imbricata* are used for the treatment of colds, coughs, lung problems, fever, pulmonary diseases and energy loss in the Tibetan and Amchi traditional medicine system [5]. The plant has also demonstrated its value because of the medicinal properties used to increase work productivity, physical endurance, longevity and to treat asthma, fatigue, impotence, hemorrhage and diseases related gastrointestinal track.

1.6 Pharmacological Value

Recent pharmacological studies have shown that the aqueous, ethanolic and hydroalcoholic roots of *R. imbricata* have an immunomodulatory and anti-cellular potential [15], immunostimulatory activity [16, 17], adjuvant activity [18], adaptogenic activity [30], radioprotective efficacy[20 - 22], radiomodulatory [22], cytoprotective [23], antioxidant potential [23], free-radical scavenging activity and metal chelating activity [22], cold, hypoxia and restraint (C-H-R) exposure and post-stress recovery [24], anti- proliferative effects [25], anti-cancerous [25], potential of healing dermal wound [26], hepatoprotective effect [27], radical scavenging and antiproliferative activity of extracts human colon cancer cells [28], and found to be safe[29 - 30].

1.7 Classification

***R. imbricata* is classified as follows**:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Rosales

Family: Crassulaceae

Genus: *Rhodiola*

Species: *imbricata Edgew*

Figure 1.1: *Rhodiola imbricata* from trans- Himalayan regions of Leh and Ladakh, India [9]

The plant has been extensively used for traditional medicines by the people of Leh and Ladakh. It also finds its mention in Tibetan and Chinese medicines but due to easy availability of *R. rosea* and *R. crenulata,* the potential of *Rhodiola imbricata* has not been completely exploited yet by the pharmaceutical industries. This species contains medicinally important secondary metabolites like Salidroside, Rosavin, Rosin and Tyrosol. But plant cell cultures usually encounter problems of low product yields and high cost, which discourage their commercialization. Moreover, the quantitative analysis and HPLC studies of these metabolites proved their concentration to be relatively less in comparison to other species. So, in order to enhance the secondary metabolite content in *R. imbricata*, certain elicitors can be used to provide stress conditions to the plant cells. In this project, we aim to study the effect of different abiotic elicitors on secondary metabolite content of this plant. It will not only serve the pharmaceutical industry on large but will also help in increasing India's hold on rosavin and salidroside production in the market due to exclusivity of *R. imbricata* in Trans-Himalayan regions of India.

So, in order to achieve this objective, five kinds of chemical elicitors and two possible precursors of salidroside synthesis were employed in this research, and their effects on the morphology of the plant along with Salidroside content were investigated. Along with this, we also employed three physical elicitors to provide additional stress conditions for the plant and similarly, their effects were also studied on the plant morphology and the content of desired secondary metabolites using HPLC techniques. These strategies can be optimized to obtain a high salidroside yield to meet the industry demand.

AIMS AND OBJECTIVES:

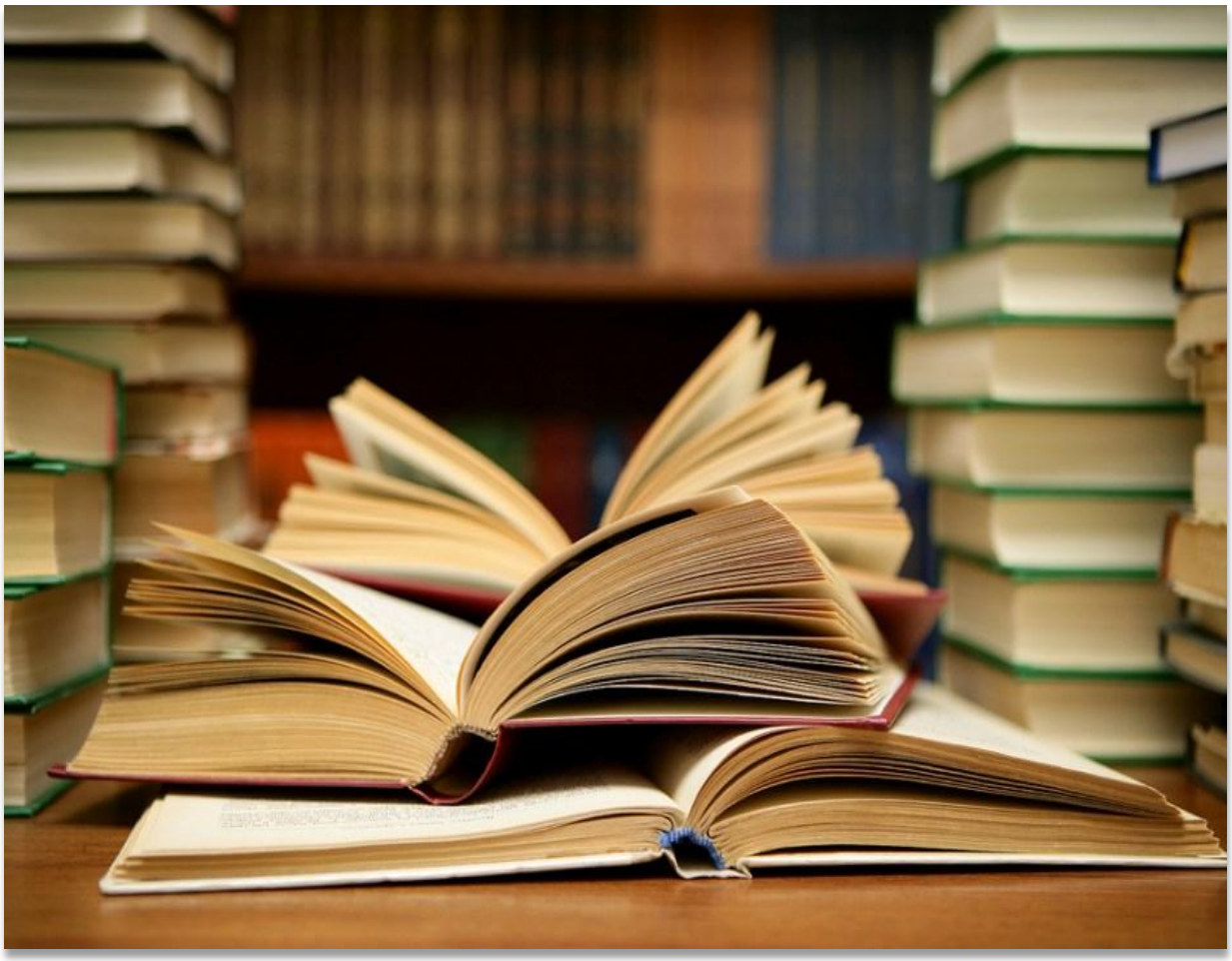
Therefore, given the strong demand for *Rhodiola* raw materials worldwide, the presence of species in the cold deserts of the Arctic and mountains which adds to cost and makes collection difficult, inherent difficulties in cultivation, over exploitation from native population, this study has been proposed to investigate the possibilities to enhance the production of bioactive compounds like salidroside. The proposed study has following objectives:

Objectives

- To study the effect of different chemical and physical elicitors in shoots and callus cultures for the production and accumulation of salidroside by quantifying it using HPLC and analyze their effects on plant's growth, development and morphology.
- Optimization of liquid media for enhancing biomass of *Rhodiola imbricata*

CHAPTER 2

REVIEW OF LITERATURE



CHAPTER 2

REVIEW OF LITERATURE

2.1 Cultivation and propagation of *Rhodiola imbricata*

For its root yield, *Rhodiola* spp. can be grown efficiently using organic plant culture methods in southern Finland according to the culture experiments [9]. For the natural stratification of winter, the seeds should be planted in the fall to produce seedlings. For approximately one year before transplant, these seedlings should be kept in pots due to the slow growth of the plants during the first 2-3 years. After four years of planting, the first root yield is harvested. The weight of the root and the yield of the roots of the plants depend to a great extent on the age.

R. imbricata plant is propagated by two methods, namely sowing seeds and the cutting of rootstocks. 65% of the germination of the seed was done in the field, while in the case of the plantation of rootstocks; 86% of the survival rate was achieved. Therefore, better rates of dispersion have been achieved through the division of rootstocks. The plants of 3-5 years are considered ideal for the plantation of appropriate rootstocks.

Under in vitro conditions, callus induction and indirect stem regeneration were found to be excellent in MS media with BAP (1 mg / L) and IBA (2 mg / L), while using MS media containing cytokinins, BAP (1 mg / l) and KN (2 mg / L) resulted in the formation and proliferation of direct shoots. MS media with BAP (2 mg / l) and IBA (4 mg / l) induced roots in vitro in 25-30 days. The survival rate of 75-80% was demonstrated by plants rooted in vitro after hardening in the greenhouse [31].

Tasheva et al. [32] developed efficient schemes for the regeneration and micropropagation of *R. rosea*. Yan et al. [34] developed a modified temporary immersion bioreactor with forced ventilation that reduces the rate of hyperhydration and improves the quality of outbreaks and multiplication rates in *R. crenulata*. . Monika et al. [33] showed a successful encapsulation of differentiated callus and axillary buds in calcium alginate beads in *R. kirilowii*. These capsules were able to grow in shoots and seedlings in solid basal MS medium

2.2 Pharmacological properties

A review of the literature has shown that in many in vitro and in vivo studies on cells and animals, the secondary metabolites of the *Rhodiola* species influence a number of physiological functions and have shown strong biological activity. These include the regulation of neurotransmitter levels, central nervous system activity and cardiovascular function. It is used to stimulate the nervous system, improve work performance, eliminate fatigue, reduce depression and prevent diseases at high altitude including altitude sickness. Most of these effects have been attributed to components such as salidrosides (rhodiolosidos), rosavinas, colofonia and p-tyrosol. Numerous pharmacological studies on *R. imbricata* have shown that this plant has adaptogenic and protective properties against stress [35-38], acts as an antioxidant [39-42], antitumor [43-45], antidepressant [46], protective activities for neurological wounds [47-48], cardioprotective, anti-inflammatory and healing dermal wounds. It has also shown to have anti-aging, immunostimulatory, radioprotective and anticancer properties [51]. Some people have used *Rhodiola sp*. to treat diabetes, tuberculosis, aging and liver damage. It also improves hearing, strengthen the central nervous system and strengthens immunity. All these reports validate their use in the traditional medicine system. In addition, in the traditional medicine system Amchi and Tibetan, the roots of *Rhodiola imbricata* are used against lung problems, colds, cough, and fever, loss of energy and lung discomfort [5].

Therefore, *Rhodiola* preparations can be applied therapeutically to humans to prevent or treat disorders such as neurodegenerative diseases, fatigue, hypoxia, cerebral ischemia, diabetes, cancer and many others.

2.2.1 Adaptogenic and antifatigue activity

Adaptogens are substances that allow the normalization of physiological responses to different stresses, increase tolerance to body stress and improve work performance [35; 56]. *Rhodiola* extracts are very useful in the treatment of aesthetic conditions that develop after intense physical or intellectual tension, which include decreased work performance, difficulty sleeping, irritability, lack of appetite, high blood pressure.

arterial pain, headache and fatigue. Therefore, it is effective at preventing oxidative stress after exhaustive exercise.

2.2.2 Elongation of lifespan and anti-aging activity

An animal study has shown that *Rhodiola* extract could inhibit T cell death, which is important since the thymus function decreases with age. It reversed the effects of aging induced by D-galactose in neuronal and immune systems, increased memory latency, improved motor activity and improved lymphocyte mitogenesis and interleukin-2 production (IL-2) [57];[60-61].

2.2.3 Antioxidant properties

Singlet oxygen scavenging, ferrous chelating, H_2O_2 scavenging, ferric reducing, hypochlorite scavenging and protein thiol protection activities were observed [62]. Salidroside reduced the intracellular production of reactive oxygen species (ROS) by hydrogen peroxide in human RBCs. Salidroside also increased cell survival and prevented human erythrocytes from suffering H_2O_2 -mediated eruption or erythrosis [63].

2.2.4 Anti-depressant

It was observed that oral administration of salidroside for 2 weeks increased the overactivity of olfactory bulbectomy in an open-field test and reduced the immobility time in a forced swimming test [65]. A reduction in the levels of TNF-α and IL-1b in the hippocampus has also been observed [65].

2.2.5 Skin Treatments

UVB-induced hyperpigmentation in brown guinea pig skin was inhibited by using salidroside by reducing the number of DOPA-positive melanocytes in the basal layer of the epidermis and reducing tyrosinase activity and melanin synthesis in melanocytes [67]. *Rhodiola rosea* extract, salidroside and tyrosol can be effective skin whitening agents.

Other physiological effects of *Rhodiola* and Salidroside include [68]

- Anti-Inflammatory
- Protection against Neuron damage
- Liver protection
- Reduction of oxidative stress in cardiovascular diseases
- Can be used in Diabetes Mellitus
- Obesity
- Anti-Viral
- Can be used against Lung Cancer

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In the phytochemical study, the content of salidroside, rosavin and its derivatives in *Rhodiola* plants depends on the morphological parts of the plant as if extracted from roots or rhizomes. In addition, it also depends on the age and sex of the plant, where the male rhizomes of the plants accumulated higher amounts of salidroside in *R. rosea* than their female counterparts [69; 70]. The location and timing of collection also influence the salidroside content of the plants [71]. To date, document data related to the chemical profile of *Rhodiola* plants have shown that samples taken from natural sources have a higher salidroside and rosin content than in the cultivated fields.

2.3 Biosynthesis of Salidroside

The biosynthetic pathway of L-tyrosol (precursor of salidroside) and the regulatory mechanism of this pathway are not completely known [72]. Salidroside is a tyrosol 8-O-b-D-glucoside [73] chemically, that is accumulated mainly in the roots and rhizomes of several species of *Rhodiola*. It is synthesized as a result of the dehydration between the glucose hemiacetal hydroxyl and the 4-hydroxyphenylethanol [74].

The literature contains two different points of view on the biosynthesis of salidrosides. Both approaches are related to the shikimic acid pathway that produces L-phenylalanine or L-tyrosol [75]. The first states that the p-coumaric acid precursor derived mainly from phenylalanine synthesizes tyrosol with a decarboxylase [76]. The second indicates that tyramine synthesized from tyrosine may be the precursor of tyrosol.

2.3.1 P-coumaric acid as a precursor

In order to clarify the molecular pathways involved in the biosynthesis of tyrosol, a study was conducted to analyze the effect of overexpression of the endogenous PALrs1 gene in *R. sachalinesis* on the production and accumulation of higher levels of p-coumaric acid and, therefore, salidroside. A 3.3-fold increase in p-coumaric acid was obtained by over expression of the PALrs1 gene. However, there was a 4.7-fold, 2.6-fold and 7.7-fold decrease in tyrosol, tyrosine and salidroside levels, respectively, compared to PALrs1 transgenic plants compared to controls. The accumulation of a higher concentration of p-coumaric acid due to the over expression of the PALrs1 gene did not facilitate the biosynthesis of the tyrosol. Therefore, it was concluded that L-phenylalanine is not the direct source of tyrosol.

2.3.2 Tyramine as a precursor

TyrDC gene encoding tyrosine decarboxylase was analyzed in *R.rosea* [77]. The expression of TyrDC gene was detected in plant leaves and roots. It was noted that the gene expression was comparitively higher in roots, due to accumulation of salidroside in the underground parts of the plant. Expression was directly dependent on amount of salidroside accumulated.

The identical results were reported when a cDNA encoding TyrDC was isolated from *R. sachalinensis* and the effects of recombinant RsTyrDC and sense and antisense over expression of endogenous RsTyrDC in tyrosol synthesis were studied. For sense-RsTyrDC expression, RNA gel blotting marked the over-expression of RsTyrDC at transcriptional levels. TyrDC activity in the RsTyrDC transgenic lines was 74% and 127 % higher, than in the empty vector-transformed control plants. This overexpression led to the accumulation of tyrosol and salidroside. Simultaneously, reduced accumulation of the endogenous TyrDC transcripts of R. sachalinensis in the antisense plant was reported.

From functional analysis, tyrosine has been shown to be TyrDC's best substrate. A marked increase in tyrosol and salidroside in *R. sachalinensis* was achieved by overexpression of TyrDC sense, suggesting that TyrDC can regulate the biosynthesis of tyrosol and salidroside, and therefore, it was concluded that TyrDC is more likely to

have a function in the first reaction of the pathway responsible for the biosynthesis of. Salidroside in *R. sachalinensis* [78].

Summing up the previous research, we can conclude and verify in our studies that decarboxylation of tyrosine by TyrDC marks the beginning of salidroside biosynthesis, which produces tyramine.

In the secondary plant metabolism, the synthesis of low molecular weight glycosides is catalysed by UDP-glucosyltransferase. The last step in the biosynthesis of salidrosides is a reaction of adding a molecule of glucose to the tyrosol molecule. It is possible that UDP-glucose transferase is the enzyme that catalyzes the final reaction using UDPG (an active and energy-rich form of glucose in living organisms) as a glucose donor and tyrosol as a receiver [74].

In terms of salidroside metabolism, β-D-glucosidase can be of a dual nature. It can participate in both anabolism and the reaction to catabolism. During the anabolism reaction, a glucosyl is transferred to tyrosol (the aglycone) and forms the salidroside, while during the catabolism, salidroside breaks into glucose and tyrosol. At present, it is unclear whether the activity of the enzyme hydrolase or transferase has priority in tissues in vivo, or whether it acts differently at different stages of development and / or different tissues and plants [74].

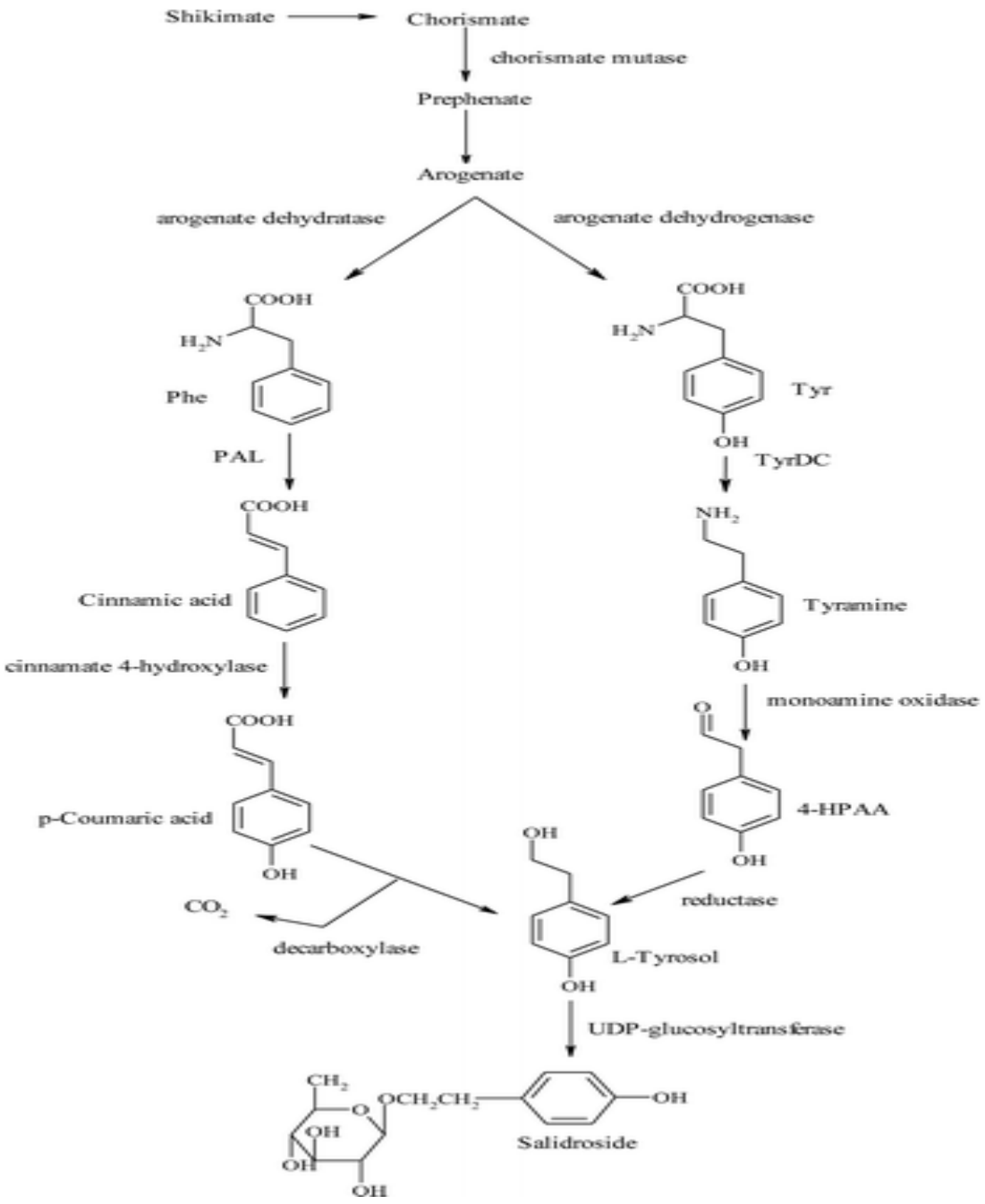


Figure 2.1: Salidroside proposed biosynthesis pathway [73].

2.4 Elicitors to enhance secondary metabolite content:

Advanced biotechnical methods offer numerous possibilities to produce efficacious compounds under in vitro controlled conditions which are independent of drastic changes in the environment. Implementation of scale-up procedures may help in reducing labour costs and improve the rate of production of secondary metabolites. In addition to this, the cells, organs and genes involved directly with the biosynthesis of compounds of interests could be cultured, mass propagated and altered to enhance their production.

An 'elicitor' is defined as a substance which, when introduced in small defined concentrations to a living cell system, triggers, initiates or enhances the biosynthesis of specific metabolic compounds, generally, secondary metabolites. Elicitation is defined as induction or enhancement of biosynthesis of metabolites due to the addition of trace defined amounts of elicitors. Though elicitation has proved to be an effective technique in enhancing secondary metabolism in plants or in vitro grown plant cells but the exact mechanism of this technique is not completely understood.

There are several shreds of evidences that show adding precursors or inducing stress to plants show a significant increase in secondary metabolite production and release. Many species of *Rhodiola* showed a gradual increase in production of rosavin and salidroside when precursor molecule or compounds were added to media. The fold change reported ranges from 3-4 fold increase.

Reports confirm that salidroside could be artificially produced under large-scale in vitro conditions [79] or could be obtained in large quantities that often exceeded their content in *Rhodiola* plants from other botanical families, for example, Ericaceae, Salicaceae.
Not only salidroside but also rosavin, rosin and rosarin were identified as important and active biological compounds in *Rhodiola* extracts, responsible to cause pharmacological effects. Furthermore, the superior efficacy of the total *Rhodiola* extract compared to salidroside alone has also been demonstrated [80].

2.4.1 Methyl Jasmonate as an elicitor

Methyl jasmonate (MeJ) and jasmonic acid (JA) are established signal molecules in biotic and abiotic stresses. Because of their application in various signaling pathways,

they have proved their worth as effective elicitors. MeJ is widely used for the production of several compounds such as alkaloids, terpenoid, phenolic phytoalexins, taxanes and coumarins in many plant species. Various studies done under in vitro conditions have proven that MeJ treatments can also activate the activity of PAL and other enzymes linked to the biosynthesis of phenols. According to a study done on *R. sachalinensis,* the administration of optimized level of methyl jasmonate, salidroside content increased by 1.5 folds [81].

Shikonin and its derivatives such as red naphthoquinone have also employed the use of MeJA for increasing its content in *Onosma paniculatum* cultured cells. Accumulation of anthocyanin induced by MeJA was reported in strawberry fruits, *Arabidopsis thaliana, Vitis vinifera* and tulip leaves. Sweet basil, *Ocimum basilicum L.*, when elicited with MeJ gave an enhanced metabolite content which was not identified through HPLC analysis. The results reported the increase of metabolite by 4.3 folds in comparison to control after induction by MeJA (0.5mM) treatment [82]

2.4.2 Pectin and Chitin as an elicitor

The interactions between plants and microorganisms involve various polysaccharides. Few of these polysaccharides such as chitosan/chitin and pectin gave the best results as elicitors by activating secondary metabolic pathways in *Morinda citrifolia* cultures [83]. The soluble fragments of chitin released by the action of chitinases on fungal cell walls act as biotic elicitors in rice (*Oryza sativa* L.*).* The above finding was efficiently used to enhance the content of diterpenes in the rice crop [84].

Pectins present in the cell wall, isolated from suspension culture (24 days old) of *M. citrifolia* when used as an elicitor has proven to give maximum production of anthraquinone[83].

2.4.3 Yeast Extract as an elicitor

A number of components present in yeast extract may be used as elicitors. The yeast cell walls comprise of chitin, manno-proteins, β -1,3- and β - 1,6-glucans whereas yeast plasmatic membrane is made up of lipids, proteins, and sterols. Most of these compounds trigger plant defense system [85]. Number of in vitro studies has reported the secondary metabolites accumulation followed by the activation of Phenylalanine ammonia lyase pathway after application of yeast extract to plant cell cultures. A study

in *R. sachalinensis* using yeast extract as an elicitor enhanced salidroside content by 3.5 folds [86].

In addition to this, according to a recent report yeast extract when applied to soybean enhanced the amount of photosynthetic pigments such as chlorophylls a, b and carotenoids. The increase in plant biomass yield, antioxidant activity and phenolic content was also reported [87].

2.4.4 L-Phenylalanine as an elicitor

As it is an established fact that L-Phenylalanine is the main precursor of Salidroside and plays an important role in numerous secondary metabolite biosynthesis from shikimate pathway, it thus forms an important elicitor. *Cistanche diserticola* cell suspension cultures when fed with Phe yielded 1.13 fold higher phenylethanoid glycosides (PeGs)[88]. Similarly, in a study performed on *R. sachalinensis*, 2 folds higher concentration of salidroside than control was obtained. Hence, it is an important elicitor to enhance salidroside production.

2.4.5 Trans-Cinnamic acid as an elicitor

The *Rhodiola* species release few common secondary metabolites but in varying concentrations. Trans- Cinnamic acid acts as an important precursor in the biosynthetic pathway of rosavin, where glycosylation of Cinnamic acid results in its synthesis. This was experimentally validated in *R.rosea* where 3 fold increase in rosavin was observed in the plants elicited with Cinnamic acid than in control. hence, it may also serve as an efficient elicitor for secondary metabolites from *Rhodiola* species in general.

2.4.6 Photosynthetic light as an elicitor

In plants, the fluctuation of morphogenic potential and biochemical responses has been strongly controlled by the exposure to light.

Various studies have been performed to check the influence of different spectral lines on plant growth, development and secondary metabolite production. In a recent study, *Stevia rebaudiana* (*S. rebaudiana*), a medicinally important plant was exposed to different wavelengths of white light. For this purpose, different coloured LED lamps were employed. According to the results obtained, white light was best suited for callus induction and increase plant biomass. Where blue light enhanced the phenolic and

flavonoid content of the plant along with increasing its anti-oxidant property, green light promoted reducing power assay [91].

Similarly, the callus cultures of *Z. officinale* when exposed to different light intensities stimulated the production of secondary metabolites such as zingiberene and gingerol [92]. Reports also suggest that the increase in light intensities also enhanced the level of phenolics.

2.4.7 Ultraviolet Radiations as an elicitor

In a recent study in apples, the wavelength of 280–320 nm of UV light when combined with red light, synergistically stimulated the biosynthesis of anthocyanin. Likewise, the exposure to UV-B irradiation increased the concentration of flavonols in Norwegian spruce (i.e. *Picea abies*). The plants of *Catharanthus roseus*, exposed to UV-B light, show significant increases in the production of vincristine and vinblastine, which have shown their effectiveness in the treatment of lymphoma and leukaemia [93].
The UV-B radiation could increase the content of flavonoid and enhance the PAL activity which leads to decrease in the chlorophyll amount. It has also been shown that UV-B induces the production of flavonols in silver birch and vine leaves. Hence, it is an established elicitor for anthocyanins, flavonoids and its derivatives.

2.4.8 Electricity as an elicitor

Electricity when tested as an elicitor in a variety of plant species, it was shown to induce the biosynthetic pathways of secondary metabolites. The reports suggest that electro-elicitation could serve as an important means to elicit the production of specific plant metabolite or a set of metabolites which are the end product of different biosynthetic pathways. In a study done on *Pisum sativum* which is non-transgenic pea hairy roots the treatment with 30 -100 mA of electric current increased the amount of pisatin by 13 folds than the control [94].

Application of electricity as an elicitor does not cause any adverse physiological changes in the cells or tissue. The use of electric current in hydroponics eliminated the requirement and cost of sacrificing the plant tissue to attain the metabolites. This elicitor has also proved its utility when used in large scale bioreactors by limiting the need to decontaminate the plant tissue as well as the desired metabolite.

2.4.9 Ultrasonication as an elicitor

Saffron *(Crocus sativus)* callus suspension culture was treated with ultrasonic waves under optimal growth conditions. The analysis of ultrasonically treated plants and the control showed a significant difference in the amounts of crocin and safranal produced. The highest concentration of safranal was achieved in suspension culture samples treated twice with ultrasound at an interval of 72 hours, and the medicinally important metabolite, crocin was maximised in samples treated twice after 24 hours duration [95].

Table 2.1: Review of different elicitors used to enhance the content of secondary metabolites in different *Rhodiola species* and other plants.

S.No	Elicitor	Plant species	Metabolite	Fold change	Reference
1.	Methyl Jasmonate	*R. sachalinensis*	Salidroside	1.5 folds	Wang Yi Wen *et al*.
2.	Pectin	*M. citrifolia*	Anthraquinone	1.7 folds	Domenburg *et al. (1994)*
3.	Chitin	*Oryza sativa* L.	Diterpenes	1.3 folds	Yue-Ying Ren *et al.*(1992)
4.	Yeast Extract	*R. sachalinensis*	Salidroside	3.5 folds	J.S. Lee *et al.* (2008)
5.	L-Phenylalanine	*R. sachalinensis*	Salidroside	2 folds	Wu et al. (2003)
6.	Cinnamic Acid	*R.rosea*	Rosavin	3 folds	Gyorgy et al. (2004)
7.	Photosynthetic Light	*Stevia rebaudiana*	Flavanoid	2.2 folds	Naveed Ahmad *et al.* (2016)
8.	Ultraviolet radiations	*Catharanthus roseus*	Vincrisine	1.7 folds	Y. K. Bernard *et al.* (2009)
9.	Electric current	*Pisum sativum*	Pisatin	13 folds	Evans Kaimoyo *et al* (2008)
10.	Ultrasound	*Crocus sativus*	Crocin and Saffranin	4-8 folds	Tofigh Tahikhani *et al.* (2017)

On careful analysis and study of the ongoing research, it was observed that *R. imbricata* has remained unexplored in terms of enhanced secondary metabolite production unlike other *Rhodiola* species. As the plant carries the plethora of pharmaceutical significance due to which the collection is reckless from its natural habitat. Owing to this fact, we have tried to enhance the production of medically sound metabolite salidroside using different elicitors such as ultrasonic waves, electric gradient, varied temperature conditions, exposure to photosynthetic light, ultraviolet radiations and chemical elicitors such as Cinnamic acid, L-phenylalanine, methyl jasmonate, yeast extract, chitin, pectin etc.

CHAPTER 3

MATERIAL AND METHODS

CHAPTER 3

MATERIAL AND METHODS

3.1 Selection of Plant Material

R. imbricata tissues were obtained from callus grown in vitro in tissue culture chambers at 15 ± 2 ° C with relative humidity of 70%, a photoperiod of 16 hours per day / 8 hours at 3000 lux of florescent tubes (Philips, India) at Jaypee University of Information Technology, Waknaghat, HP, India (Figure 3.1).

Figure 3.1 *R. imbricata* in vitro grown callus in plant tissue culture chambers of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India.

3.2 Optimization of Solid growth media and culture conditions

Various MS media combinations were tested for optimization of the culture condition for micropropagation of *R.imbricata.* Different concentrations and combinations of 6-Benzylaminopurin (BAP), Indole-3-butyric acid (IBA), Kinetin (KN), Gibberellic acid (GA_3) and Thidiazuron (TDZ) with sucrose 3% (w/v) were supplemented with MS media. The pH of the each media was adjusted to 5.7 with 10 N HCl and NaOH and 0.8% (w/v) of agar-agar (Plant tissue culture grade) was added as a gelling agent and they were autoclaved at 121°C temperature and 15 lb/in pressure for 15 - 20 min. The cultures were incubated in plant tissue culture chambers maintained at incubation temperatures 15 ± 2 ° C with relative humidity of 70%, a photoperiod of 16 hours per day / 8 hours at 3000 lux of florescent tubes (Philips, India) at Jaypee University of Information Technology, Waknaghat, HP, India (Figure 3.1).

Table 3.1: MS media supplemented used for callus induction and shoot regeneration in *Rhodiola imbricarta*.

S.No.	Medium Name	MS media composition
1.	AA0	MS
2.	AA1	MS + BAP (2 mg/L) + KN (2 mg/L)
3.	AA2	MS + TDZ (1 mg/L)
4.	AA3	MS + BAP (1 mg/L) + IBA (2 mg/L)
5.	AA4	MS + IBA (1 mg/L) + 2,4-D (1.5 mg/L)
6.	AA5	MS + BAP (0.5 mg/L) + IBA (2 mg/L) + GA_3 (2 mg/L)
7.	AA6	MS + IBA (4 mg/L)
8.	AA7	MS + BAP (1 mg/L) + KN (2 mg/L)
9.	AA8	MS + KN (2 mg/L) + IBA (1 mg/L)
10.	AA9	MS + BAP (2 mg/L) + GA_3 (2 mg/L)

3.3 Establishment of Axenic cultures

Regeneration of callus and direct shoot organogenesis were carried out from in grown callus culture incubated at 15 ± 2°C and 25 ± 2°C in plant tissue culture chambers**.**

3.3.1 Callus Formation

Callus formation was carried by transferring finely excised small sized calli on different media combinations (Table 3.1) under above mentioned culture conditions. Data were recorded on days to initiation of callus growth, callus morphology and percentage of calli. These experiments were performed in triplicates and repeated three times. Subculturing was carried out after every 15 – 20 days for 2 months so as to obtain good growth.

3.3.2 Shoot regeneration from callus

After the growth of callus, MS media consisting of different auxins and cytokinins (Table. 3.1) were tested for shoot regeneration under aseptic conditions as mentioned above. The data on days to shoot regeneration, average shoot numbers and shoot length (cm) were recorded. These experiments were performed in triplicates and repeated thrice.

3.4 Elicitations of *Rhodiola imbricata* cultures

In vitro grown shoots and callus were transferred to flasks containing a set of physical and chemical elicitors and were incubated in 15 ± 2°C plant tissue culture chambers (Table 3.2).

3.4.1 Chemical Elicitors

3.4.1.1 Cinnamic Acid

The in-vitro grown shoots and callus have been cultured on MS media supplemented with growth hormones and different concentrations of Cinnamic acid (1.0 mM/L, 1.5 mM/L and 2 mM/L) and incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.1.2 Chitin

The in-vitro grown shoots have been cultured on MS media supplemented with growth hormones and different concentrations of Chitin (0.5 g/L, 1.5 g/L and 2.0 g/L) and incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.1.3 L- Phenylalanine

The in-vitro grown shoots have been cultured on MS media supplemented with growth hormones and different concentrations L- Phenylalanine (0.5 mM/L, 1 mM/L and 2

mM/L) and incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.1.3 Pectin

The in-vitro grown shoots have been cultured on MS media supplemented with growth hormones and different concentrations of Pectin (0.5 g/L, 1.5 g/L and 2.0 g/L) and incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.1.4 Methyl Jasmonate

The in-vitro grown shoots have been cultured on MS media supplemented with growth hormones and different concentrations of Methyl Jasmonate (0.25 mM/L, 0.5 mM/L and 1 mM/L) and incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.1.5 Yeast Extract

The in-vitro grown shoots have been cultured on MS media supplemented with growth hormones and different concentrations of Yeast Extract (0.5 g/L, 1.0 g/L and 2.0 g/L) and incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

Table 3.2 : Chemical components used as elicitors along with their tested concentrations in *R.imbricata*.

S.No	Elicitor (Chemical)	Concentrations Tested
1	Cinnamic Acid	1.0 mM/L, 1.5 mM/L and 2 mM/L
2	Chitin	0.5 g/L, 1.5 g/L and 2.0 g/L
3	L- Phenylalanine	0.5 mM/L, 1 mM/L and 2 mM/L
4	Pectin	0.5 g/L, 1.5 g/L and 2.0 g/L
5	Methyl Jasmonate	0.25 mM/L, 0.5 mM/L and 1 mM/L
6	Yeast Extract	0.5 g/L, 1.0 g/L and 2.0 g/L

3.4.2 Physical Elicitors

3.4.2.1 Electric Shocks

Finely excised shoots submerged in MS media were induced to electric shocks using a DC voltmeter at 5 mA, 10 mA, 20 mA, 30 mA, 50 mA, 75 mA, 100 mA, 125mA, 150mA, 200mA for continuous 2 minutes. The current was measured using a Amp meter. These cultures were later transferred to solid MS media incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period (Set Up Figure 3.2).

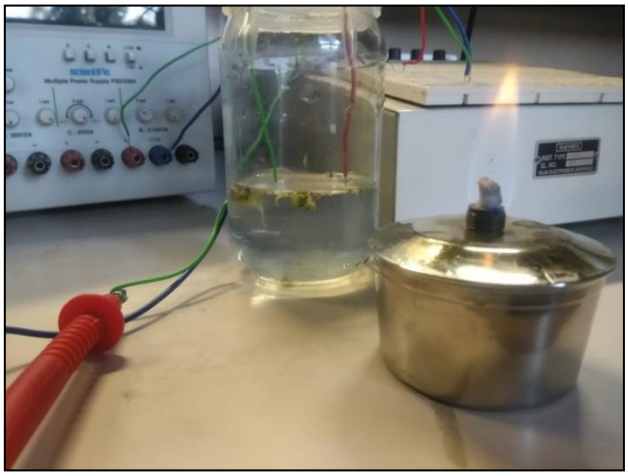


Figure 3.2: Set up used for inducing electric shock to plants

3.4.2.2 Photosynthetic Lights

Plants transferred on MS media were kept in incubation in a incubater shaker (provided by New Brunswick) integrated with Photosynthetic Growth Lamp of 4000 lux intensity at 25 ± 2°C. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.2.3 Ultrasonic Waves

Plants transferred in liquid media were induced to ultrasonic waves at 20,000 Hz for 1 minute, 1 minute 30 seconds, 2 minutes with 5 seconds rest and 10 seconds running time. The plantlets were later transferred to solid MS media incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

3.4.2.4 Ultraviolet Light

Finely excised plantlets were exposed ultraviolet light kept at 30 cm above in a closed chamber for 10 minutes, 20 minutes, 30 minutes. These plantlets were later transferred to solid MS media incubated at 15 ± 2°C plant tissue culture chambers in conditions mentioned above. The data have been collected for shoot length, number of shoots, leaves size, number of leaves, shoot biomass and salidroside concentration. These experiments were performed in triplicates and repeated thrice. Data was collected after 4 weeks of incubation period.

Table 3.3: Physical factors used as elicitors with their varying ranges in *R.imbricata*.

S.No	Elicitor (Physical)	Tested factors
1	Electric Shocks	50 mA - 200mA for 2 minutes
2	Photosynthetic Light	4000 Lux Intensity
3	Ultrasound	20,000 Hz for 1 minute, 1 minute 30 seconds, 2 minutes
4	Ultraviolet Rays	10 minutes, 20 minutes, 30 minutes

3.5 Optmization of Liquid Media for callus growth and regeneration

Integrated MS media with different concentrations and combinations of indole-3-butyric acid (IBA), 6- Benzylaminopurine (BAP), gibberellic acid (GA3), kinetin (KN) and Thidiazuron (TDZ) with 3% sucrose (w\v) observed for callus growth. The pH of each medium was adjusted to 5.7 with 10 N HCl and NaOH and sterilized in an autoclave at 121 ° C and 15 lbs/in pressure for 15-20 minutes.

3.5.1 Callus induction in liquid media

Fine excisions were made on callus, leaves and shoots of *R .imbricata* and were transferred in MS media with different growth hormone concentrations (Table 3.4). The cultures were incubated in plant tissue culture chambers maintained at incubation temperatures (25 ± 2°C) having 70 % relative humidity, 16 h day and 8 h night photoperiod at 3000 lux photosynthetic photon flux density provided by cool white fluorescent tubes (Philips, India) on a 120 rpm shaker. Biomass of callus was regularly monitored after duration of 1 week.

Table 3.4: Different liquid media composition for callus induction and regeneration in *R.imbricata* tissue samples.

S.No.	Medium Name	MS media composition
1.	AAL0	MS
2.	AAL1	MS + BAP (1 mg/L) + KN (1 mg/L) + TDZ (0.5mg/L)
3.	AAL2	MS + TDZ (1 mg/L)
4.	AAL3	MS + BAP (1 mg/L) + IBA (2 mg/L)
5.	AAL4	MS + IBA (1 mg/L) + 2,4-D (1.5 mg/L)
6.	AAL5	MS + BAP (0.5 mg/L) + IBA (2 mg/L)
7.	AAL6	MS + IBA (0.5 mg/L) + IBA (2 mg/L) + KN (2mg/L)

3.6 Quantification of Salidroside in *Rhodiola imbricata* tissues

3.6.1 Standard Preparation

A standard stock solution was prepared by dissolving 1 mg of Salidroside (Chromadex) in 1 ml of 80% methanol. These stock solutions were diluted twice to prepare 50 ppm standard working solutions and stored in an HPLC vial at 4 ° C.

3.6.2 Sample Preparation

Fresh tissues in vitro grown in vitro control and tested shoots and callus were taken for salidroside analysis were collected and stored at -80 ° C. Approximately 100 mg of crushed tissue from each sample was collected and filtered with 100% 1:15 methanol (w / v). The Sonicator water bath was used to incubate the mixture at 30 ° C for 15 minutes. The mixture was filtered through a 0.2 μm filter apparatus and the resulting extract was stored in HPLC vials at 4 ° C.

3.6.3 Chromatography Conditions

The analyzes were performed using a Waters HPLC system, equipped with HPLC 515 water pumps, a Waters 717 automatic sampler, a Waters 2996 photodiode array detector and the Empower software. The stationary phase used was the Waters Spherisorb reverse phase C18 column (4.6 mm x 250 mm, 5 µm). The temperature of the column oven was adjusted to 25 ° C. Various mobile phase compositions (methanol, acetonitrile and Milli Q water at pH 5.8) with different flow rates were tested to resolve the standard Salidroside mixture. The diode array detector was used to detect the salidroside (at 225 nm) while the injection volume was maintained at 10 µl. The peak area data and the salidroside retention time were recorded. These experiments were performed in triplicate and repeated three times. The concentration (mg / g) of the compounds was calculated using the formula:

(PA of sample/PA of standard) x (1/IV) x (Volume of sample/weight of sample) x dilution factor

Where, PA – Peak Area

IV – Injection Volume

CHAPTER 4

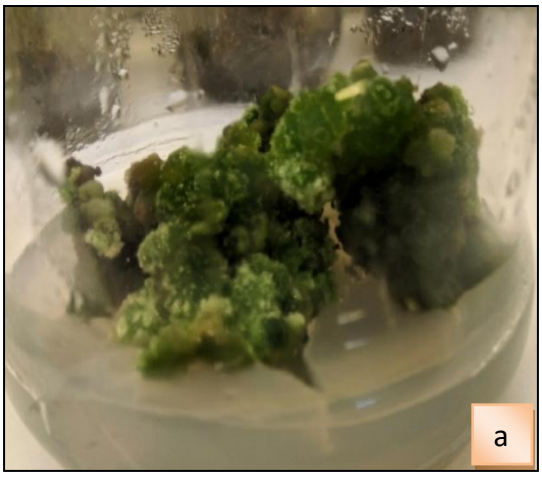
RESULTS

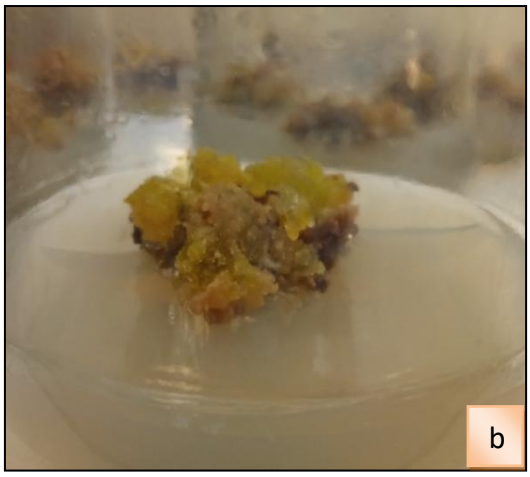
CHAPTER 4

RESULTS AND DISCUSSION

4.1. Establishment of Callus culture

Out of tested 10 media combinations (Table 4.1), callus growth was observed in 4 media combinations *viz.* AA2, AA3, AA4, AA8 at 15 ± 2°C and 25± 2°C. Callus growth was initially observed at 15 ± 2°C (10-30 days) as compared to 25± 2°C (20 -50 days). MS medium supplemented with TDZ (1 mg/L) was found to be the best for regeneration of callus within 10 -15 days at 15 ± 2°C with 81-92% of calli from old callus as well as excised leaves and shoots. Leaf explant was found to be the best for initiation of callus in 10-15 days with 91±0.67% of calli. Within 4 weeks of culture, complete callus mass was obtained from leaf explants. The callus mass was maintained by subculturing on AA2 after 4 -5 weeks. Regeneration was initiated from calli with a green appearance (Figure 4.1).

. 



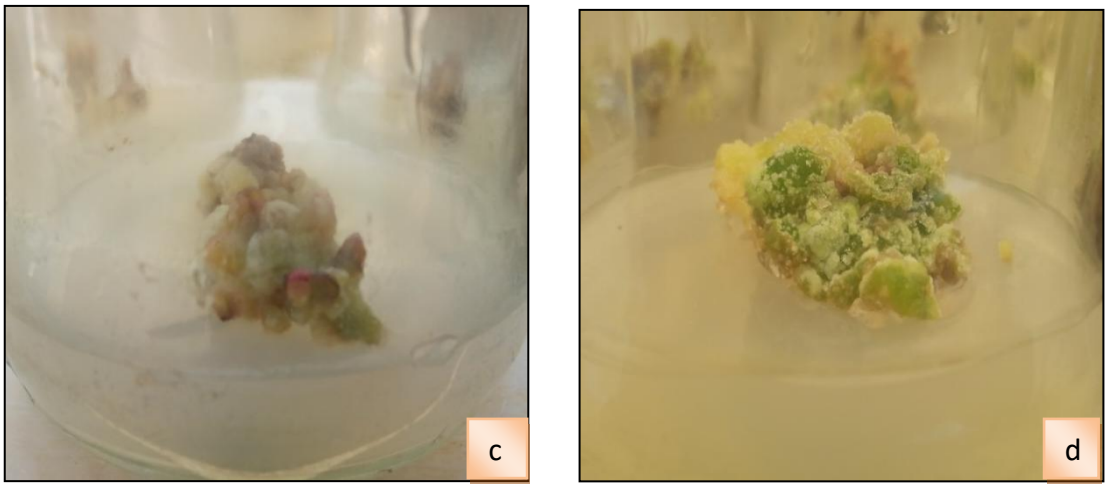


Figure 4.1: *R.imbricata* callus grown in different media composition (a) MS media + TDZ (1 mg/L) (b) MS media + BAP (1 mg/L) + IBA (2 mg/L) (c) MS + IBA (1 mg/L) + 2,4-D (1.5 mg/L) (d) MS + KN (2 mg/L) + IBA (1 mg/L)

Table 4.1: Effect of MS media supplemented with different growth hormones on callus growth and development in *R. imbricata*.

S.No.	Medium Name	Callus Growth (Days)	Callus Color	Percent Survival
1.	AA0	30 - 35	Pale Green	30±0.34 %
2.	AA1	--	--	--
3.	**AA2**	**7 - 15**	**Dark Green**	**91±0.67 %**
4.	AA3	20 - 25	Pale Green	75±0.4 %
5.	AA4	18 - 25	Creamy	45±0.5 %
6.	AA5	--	--	--
7.	AA6	--	--	--
8.	AA7	--	--	--
9.	AA8	21 - 30	Yellowish Green	65±0.5 %
10.	AA9	--		--

4. 2 Regeneration of shoots from callus

Out of tested 10 media combinations (Table 4.2) used to subculture growing callus cultures, callus was regenerated into shoots in 3 media combination *viz.* AA1, AA3, and AA7 at 15 ± 2°C and 25± 2°C. Shoot regeneration was observed initially at 15 ± 2°C (15-20 days) as compared to 25± 2°C (30-40 days). MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l) was found to be the best for shoot regeneration from calli within 15 - 20 days at 15 ± 2°C with 15.22±0.01 shoot number and average shoot length of 2.5±0.01 (Figure 4.2).

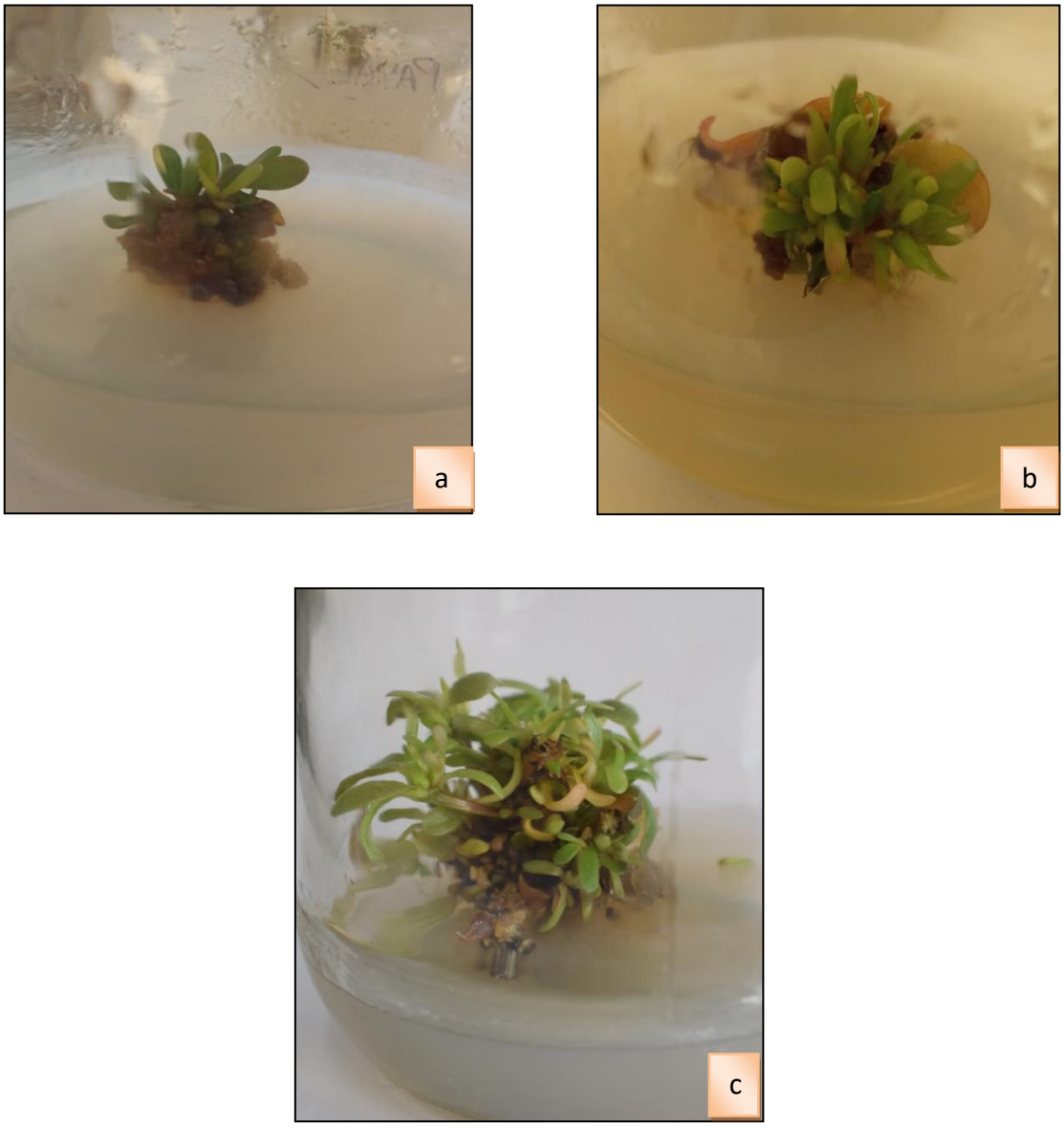


Figure 4.2: Regeneration of shoots from callus of *R.imbricata* (a) After 7 days (b) After 15 days (c) After 30 days of incubation in 15 ± 2°C plant tissue culture chambers.

Table 4.2: Shoots induction from callus in MS media supplemented with different growth hormones in *R. imbricata* .

S.No.	Medium Name	No. of Days for Regeneration	No. of Shoots
1.	AA0	--	--
2.	AA1	23 - 28	4±0.5
3.	AA2	--	--
4.	**AA3**	**25 - 30**	**8±0.75**
5.	AA4	--	--
6.	AA5	--	--
7.	AA6	--	--
8.	AA7	18 - 25	3±0.5
9.	AA8	20 - 25	4±0.75
10.	AA9	--	--

4.3 Shoot multiplication

Regenerated shoots of callus were transplanted in a different combination of media (Table 4.3). Of the 10 media combinations tested, the shoot was multiplied by 3 media combinations AA1, AA3 and AA7 at 15 ± 2 ° C and 25 ± 2 ° C. The multiplication of shoots was observed before at 15 ± 2 ° C (18-23 days) compared to 25 ± 2 ° C (25-30 days). Furthermore, at 15 ± 2 ° C, the number and duration of the outbreaks were much greater. The MS media supplemented with BAP (1 mg / L) + KN (2 mg / L) was the best for propagating shoots in 18-23 days with 5.74 shoots and an average length of 3.22 at 15 ± 2 ° C (Figure 4.3).

Figure 4.3: (a) Initiation of multiple shooting in *R.imbricata* (b) Multiple shooting in *R.imbricata* after 7 days (c) Multiple Shooting after 30 days of incubation in 15 ± 2°C plant tissue culture chambers.

4.4. Elicitations in *Rhodiola imbricata* tissues

4.4.1. Cinnamic Acid

The shoots were grown in MS media supplemented with different concentration of Cinnamic Acid i.e. 1.0 mM/L, 1.5 mM/L and 2 mM/L. The best shoot growth observed in media containing **1.5mM/L** concentration of cinnamic acid with respect to Number of Shoots **3±0.67**, Average shoot size **3±0.81 cm**, Number of Leaves **20±0.83**, Leaf size **1±0.22 cm**, Biomass **7±0.58 g** with **Dark green** color leaves (Figure 4.4, Table 4.3).

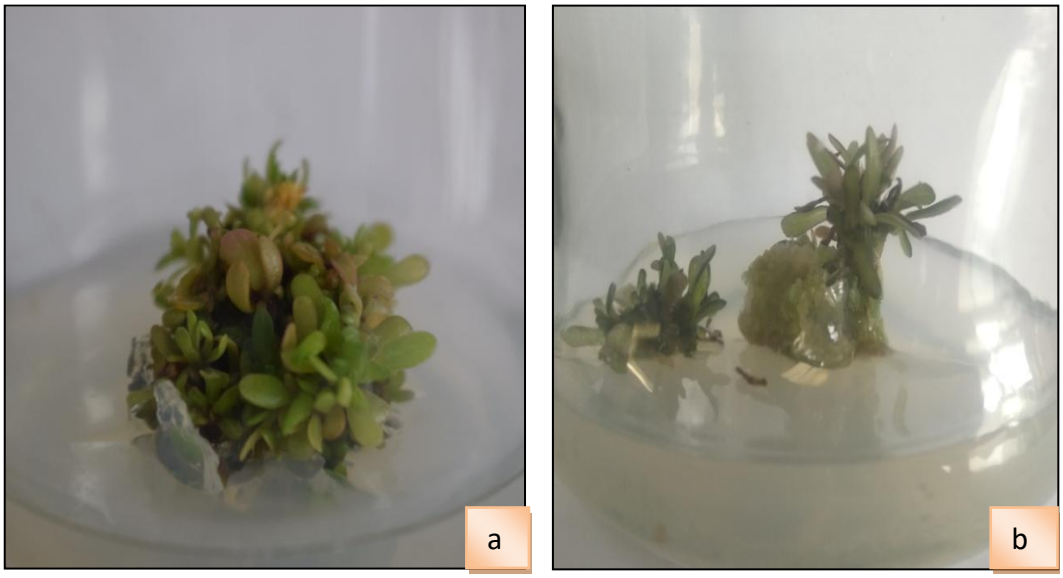


Figure 4.4: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with 1.5mM/L of Cinnamic Acid

4.4.2 Chitin

The shoots were grown in three different concentration of Chitin i.e. 0.5 g/L, 1.5 g/L and 2.0 g/L supplemented in optimized MS media. Chitin concentration (**2.0 g/L)** containing media gave best result with respect to Number of Shoots **1±0.67**, Average shoot size **0.7±0.05 cm**, Number of Leaves **5±0.16**, Leaf size **0.5±.032 cm**, Biomass **5±0.49 g** with **Red and Green** color leaves (Figure 4.5, Table 4.3).

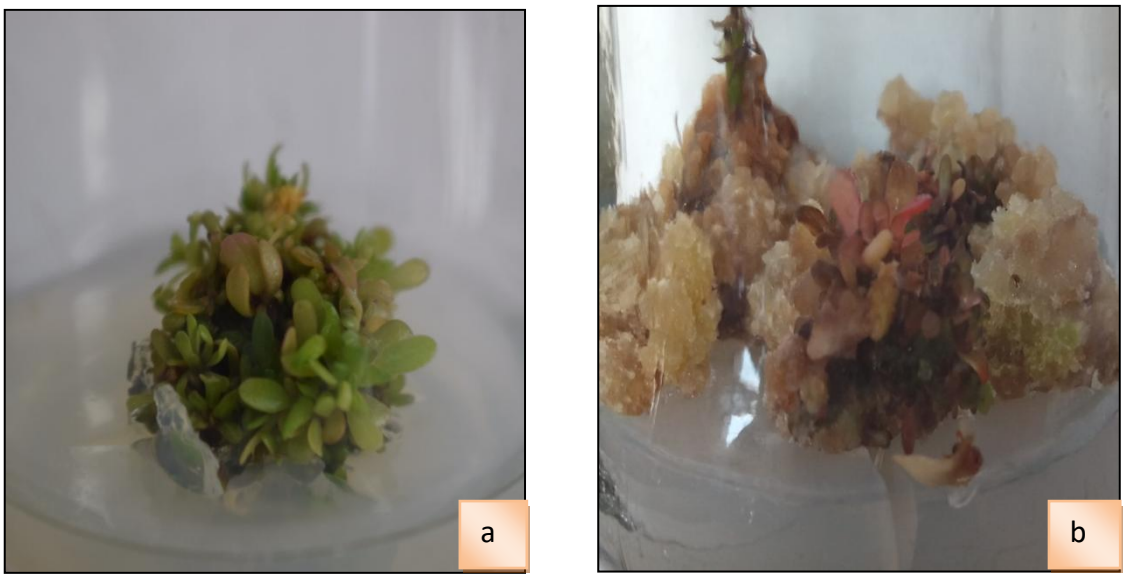


Figure 4.5: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with 2 g/L of Chitin

4.4.3 L- Phenylalanine

Three different concentration of L- Phenylalanine i.e. 0.5 mM/L, 1 mM/L and 2 mM/L were supplemented in optimized MS media. Best results were obtained in media containing shoots grown in **1.0 mM/L** concentration with respect to Number of Shoots **1±0.16**, Average shoot size **0.3±0.06 cm**, Number of Leaves **3±0.83**, Leaf size **1±.033 cm**, Biomass **0.7±0.05 g** with **Pale yellow or White dead** leaves (Figure 4.6, Table 4.3).

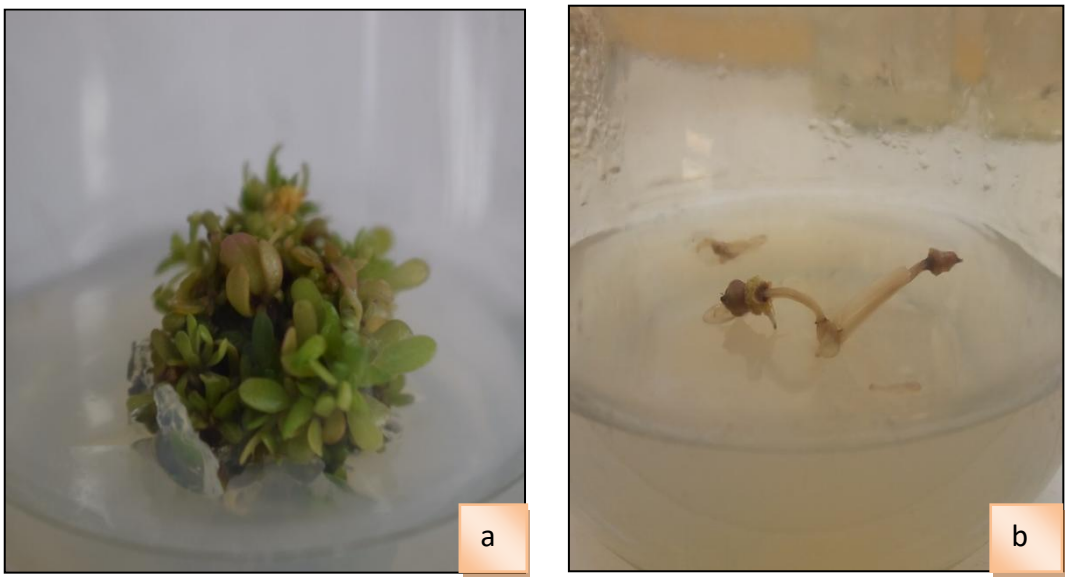


Figure 4.6: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with 1mM/L of L- Phenylalanine

4.4.4 Pectin

Pectin was also added to optimized MS medium in three different concentrations i.e. 0.5 g/L, 1.5 g/L and 2.0 g/L. Shoots grown in concentration **2.0 g/L** of pectin in MS media gave best result with respect to Number of Shoots **2±0.34**, Average shoot size **0.8±0.05 cm**, Number of Leaves **11±0.5**, Leaf size **0.9±.062 cm**, Biomass **6±0.67 g** with **Red, Green and Brown** color leaves (Figure 4.7, Table 4.3).

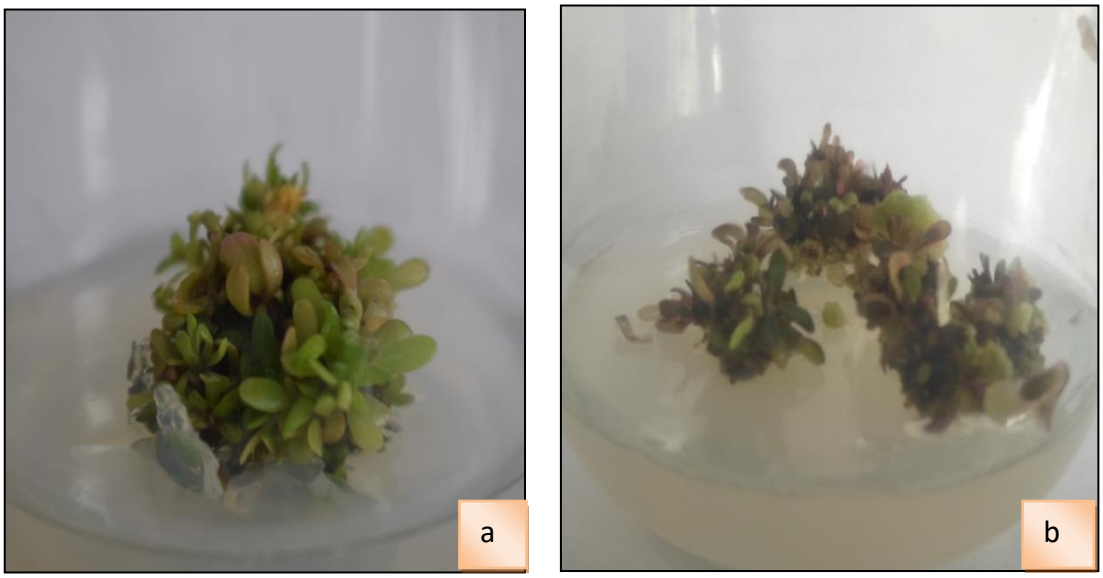


Figure 4.7: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with 2 g/L of Pectin

4.4.5 Methyl Jasmonate

The shoots were grown in three different concentration of Methyl Jasmonate i.e. 0.25 mM/L, 0.5 mM/L and 1 mM/L, supplemented in optimized MS media. The best shoot growth was observed in **0.5 mM/L** concentration of methyl jasmonate added to optimized MS medium with respect to Number of Shoots **1±0.5**, Average shoot size **0.5±0.06 cm**, Number of Leaves **12±0.16**, Leaf size **1±.033 cm**, Biomass **5±0.59 g** with **Green, Red, Brown** color leaves (Figure 4.8, Table 4.3).

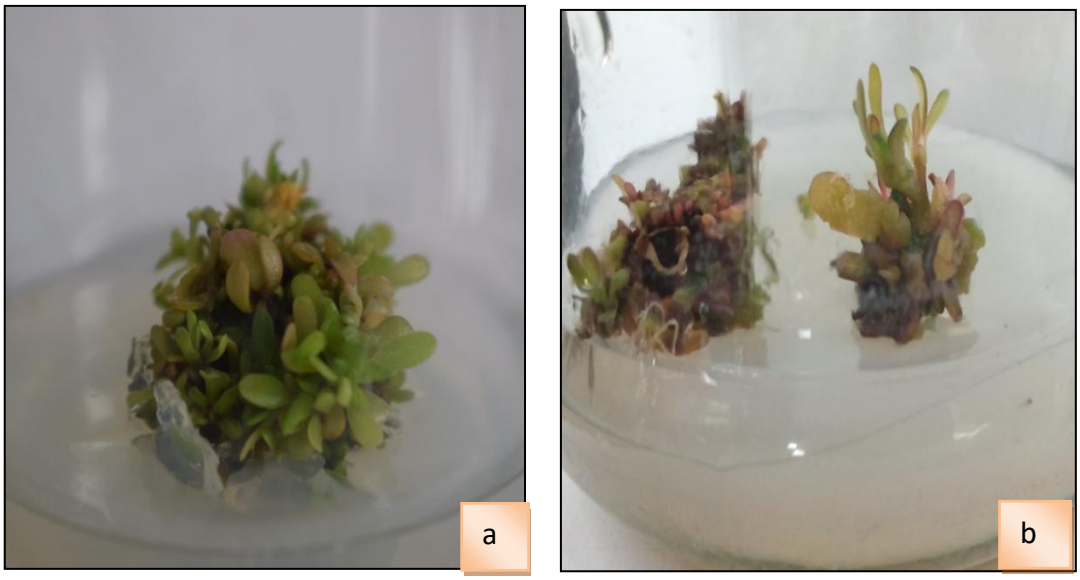


Figure 4.8: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with 0.5 mM/L of Methyl Jasmonate.

4.4.6 Yeast Extract

Optimized MS media supplemented with different concentrations of Yeast extract i.e. 0.5 g/L, 1.5 g/L and 2.0 g/L, was used for growing *R. imbricata* shoots. **2.0 g/L** concentration of YE supplemented in MS media was found to be the best with respect to Number of Shoots **1±0.16**, Average shoot size **3±0.05 cm**, Number of Leaves **8±0.5**, Leaf size **1±.033 cm**, Biomass **5±0.24 g** with **Red and Brown** color leaves (Figure 4.9, Table 4.3).

Figure 4.9: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with 2 g/L of Yeast Extract

Table 4.3: Morphological effects of chemical elicitors on in - vitro grown shoots of *R.imbricata*

S. No	Conc. Tested	Conc. Optimized	No. of Shoots	Avg. Shoot size (cm)	No. of Leaves	Average leaf size (cm)	Biomass (g)	Leaf Color
1	1.0 mM/L, 1.5 mM/L and 2 mM/L	1.5mM/L	3±0.67	3±0.81	20±0.83	1±0.22	7±0.58	Dark Green
2	0.5 g/L, 1.5 g/L and 2.0 g/L	2.0 g/L	1±0.67	0.7±0.05	5±0.16	0.5±0.032	5±0.49	Red, Green
3	0.5 mM/L, 1 mM/L and 2 mM/L	1.0 mM/L	1±0.16	0.3±0.06	3±0.83	1±0.033	0.7±0.05	Pale green
4	0.5 g/L, 1.5 g/L and 2.0 g/L	2.0 g/L	2±0.34	0.8±0.05	11±0.5	0.9±0.062	6±0.67	White, red
5	0.25 mM/L, 0.5 mM/L and 1 mM/L	0.5 mM/L	1±0.5	0.5±0.006	12±0.16	1±0.33	5±0.59	Green, red, brown
6	0.5 g/L, 1.0 g/L and 2.0 g/L	2.0 g/L	1±0.16	3±0.05	8±0.5	1±0.033	5±0.244	Red, brown

4.4.7 Electric Shocks

R. imbricata were grown in MS media after being introduced to electric shock of 5 mA, 10 mA, 20 mA, 30 mA, 50 mA, 75 mA, 100 mA, 125mA, 150mA, 200mA for continuous 2 minutes. Plants introduced to 150 mA of current for 2 minutes responded with maximum number respect to Number of Shoots **2±0.16**, Average shoot size **1±0.03 cm**, Number of Leaves **4±0.85**, Leaf size **3±.053 cm**, Biomass **5±0.96 g** with **Green** color leaves (Figure 4.10, Table 4.4).

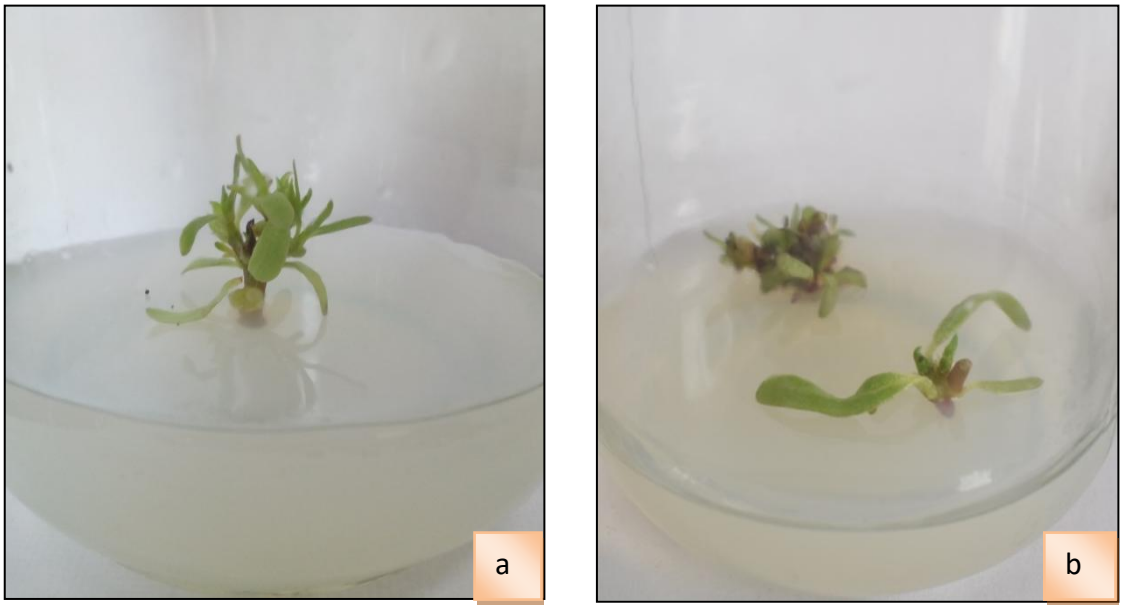


Figure 4.10: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* after inducing 150 mA of electric shock.

4.4.8 Photosynthetic Lights

Plants grown in MS media were transferred to photosynthetic growth light chamber, where some significant observations were observed with respect to shoot growth and shoot biomass. The plant otherwise grows as whorled but photosynthetic light incubation introduced elongation in internodes and changed the morphology of the plant (Fig 4.12. (c)). Other parameters were also recorded where we found maximum number of Shoots **8±0.33**, Average shoot size **6±0.63 cm**, Number of Leaves **31±0.16**, Leaf size **1±0.28 cm**, Biomass **10±0.96 g** with **Green** color leaves as compared to the control cultures which were incubated in optimized white fluorescent light. (Figure 4.11, Table 4.4).

Figure 4.11 (a) In vito grown control shoots of *R. imbricata* in normal white fluorescent light (b) Elicited shoots of *R. imbricata* in Photosynthetic Light Chamber after 15 days. (c) Fully grown shoot of *R. imbricata* in photosynnthetic light chamber after 35 days.

4.4.9 Ultraviolet Light

Plants were also tested for growth under optimized MS media after being kept under UV lamp for 10 minutes, 20 minutes, and 30 minutes. Plants kept for 30 minute of UV exposure in optimized MS media showed best result with respect to Number of Shoots **4±0.16**, Average shoot size **2±0.83 cm**, Number of Leaves **3±0.83**, Leaf size **0.9±0.063 cm**, Biomass **4±0.13 g** with **Green and Red** color leaves (Figure 4.12, Table 4.4).

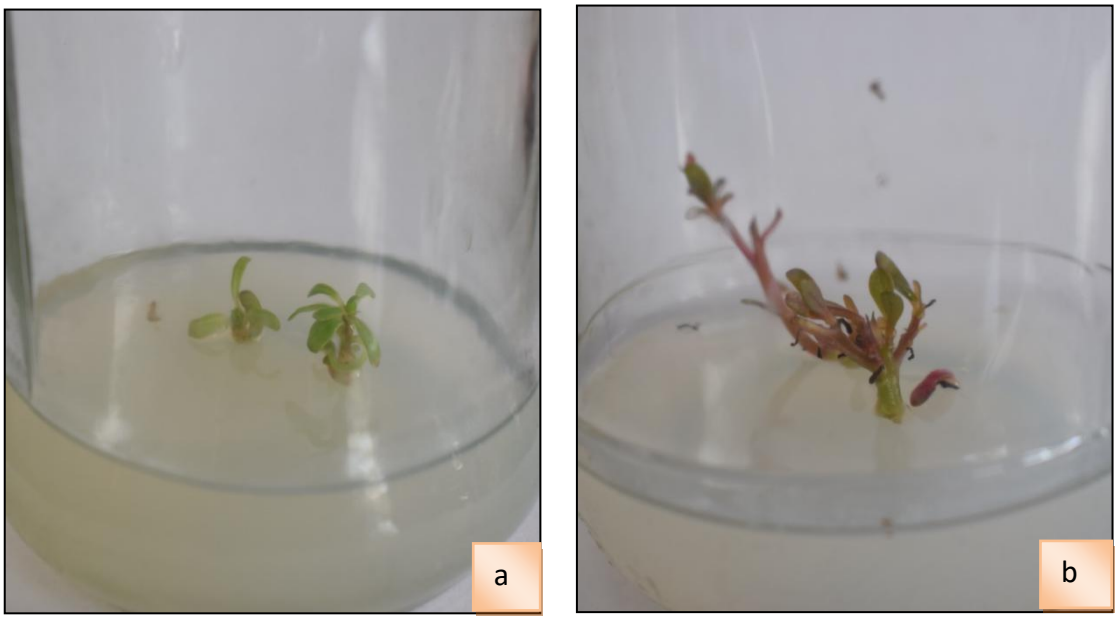


Figure 4.12: (a) In vito grown control shoots of *R. imbricata* and (b) elicited shoots of *R. imbricata* with UV exposure of 30 minutes after 10 days.

Table 4.4: Morphological effects of Physical elicitors on in - vitro grown shoots of *R.imbricata*

S.No	Elicitor (Physical)	Tested factors	Optimized Conditions	No. of Shoots	Avg. Shoot size (cm)	No. of Leaves	Avg. leaf size (cm)	Biomass (g)	Leaf Color
1	Electric Shocks	50 mA - 200mA for 2 minutes	150 mA for 2 Minutes	2±0.16	1±0.03	4±0.85	3±0.053	5±0.96	Green
2	Photosynthetic Light	4000 Lux Intensity	--	8±0.33	6±0.63	31±0.8	1±0.28	10±0.96	Green
3	Ultrasound	20,000 Hz for 1 minute, 1 minute 30	--	--	--	--	--	--	--
4	Ultraviolet Rays	10 minutes, 20 minutes,	30 Minutes	4±0.16	2±0.83	3±0.83	0.9±0.063	4±0.13	Green, Red

4.5 Optimization of Liquid Media for callus growth and regeneration

Out of tested 7 media combinations (Table 4.5), callus was regenerated in 3 media combinations *viz.* AAL3, AAL5, AAL6 at 25 ± 2°C shaker. MS medium supplemented with BAP (1 mg/L) + IBA (2 mg/L) was found to be the best for initiation and growth of callus within 10 -1 5 days at 25 ± 2°C. Green callus was observed within 15 days without browning of medium and good yield with respect to biomass was collected that is (87±0.95 g) from initial biomass of sub cultured callus 0.43±0.03.Liquid media significantly enhanced plant biomass by 203.1 fold. The average callus biomass obtained in solid media is 16±0.43 g. Thus, the relative enhancement achieved in liquid media in comparison to solid media is 5.35 folds more. The regenerated shoots 21 in number from optimized liquid cultures would used for further multiplication and up scaling experimentation. (Figure 4.13)

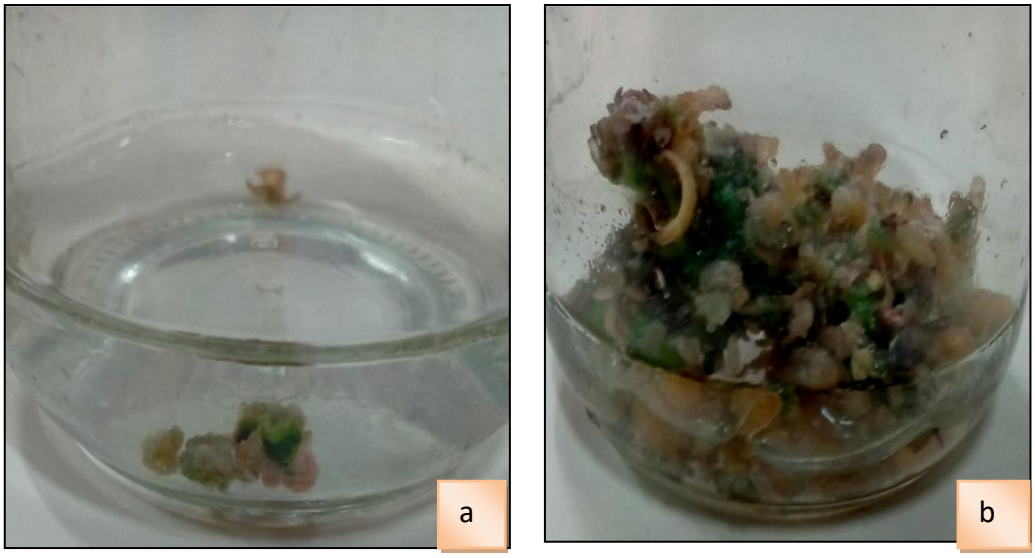


Figure 4.13: (a) Callus Growth in Liquid media after 5 days (b) Callus Growth in Liquid media after 3 weeks.

Table 4.5: Liquid Media optimized with respect to days of regeneration and Biomass yield of *R.imricata*

S.No.	Medium Name	Days For Regeneration	Biomass (g)
1.	AAL0	28 - 40	8±0.93
2.	AAL1	27 - 35	19±0.67
3.	AAL2	--	--
4.	**AAL3**	**20 – 25**	**87±0.95**
5.	AAL4	--	--
6.	AAL5	23 - 30	39±0.38
7.	AAL6	25 - 31	21±0.76

4.6 Quantification of Salidroside in *Rhodiola imbricata* tissues

Various mobile phase compositions (methanol, MilliQ water and acetonitrile of different pH values) were used based on standard salidroside assays. The optimum resolution could be achieved using MilliQ Water (B), and Acetonitrile (A) as mobile phase with isocratic elution: 15A/85B for Salidroside with flow rate of 10 mL min^{-1}. Salidroside standard was quantified with peak area of 1732268 ± 0.28 and retention time of 1.96±0.01 minutes (Figure 4.14). The chromatogram elaborated for the quantification of salidroside in standard and in all elicited shoots were mentioned in Figure 4.14, 4.15(a)-(l). The quantitative amounts of salidroside in all samples were calculated according to the formula mentioned in the material and the concepts represented in table 4.6.

4.6.1 Standard Curve

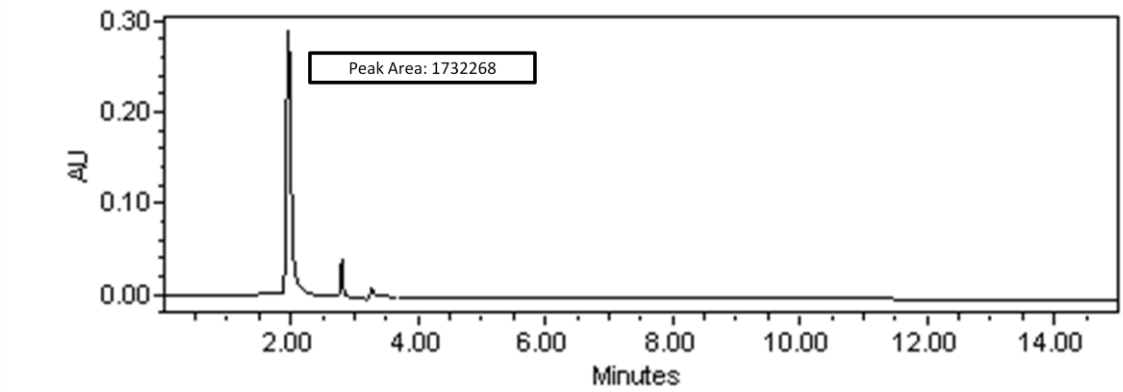
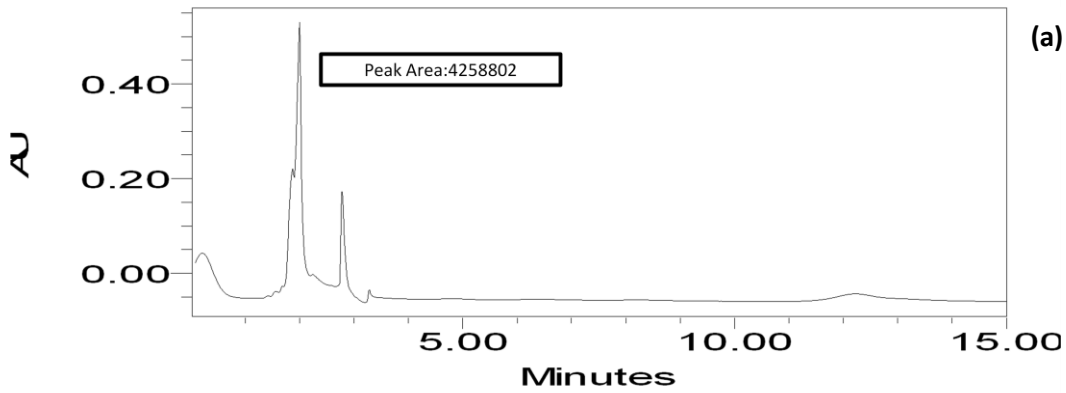


Figure 4.14: HPLC Chromatogram for Salidroside Standard.

4.6.2 Sample Curve

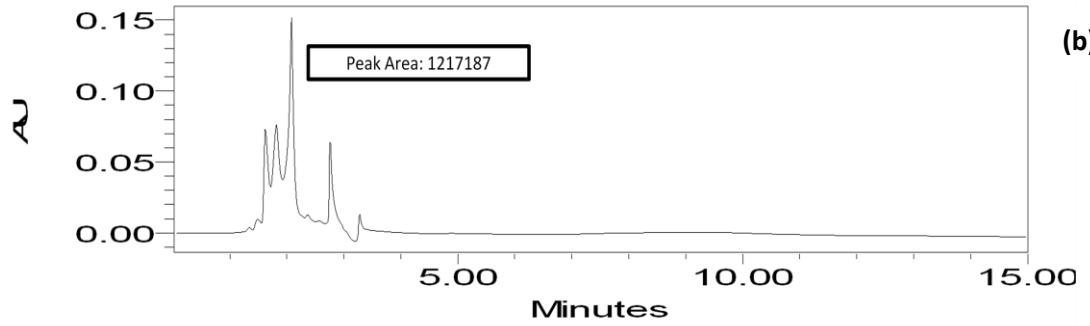
1. Shoot (15±1°C)



(a)

Salidroside Quantified: 3.688 ± 0.07 mg/g

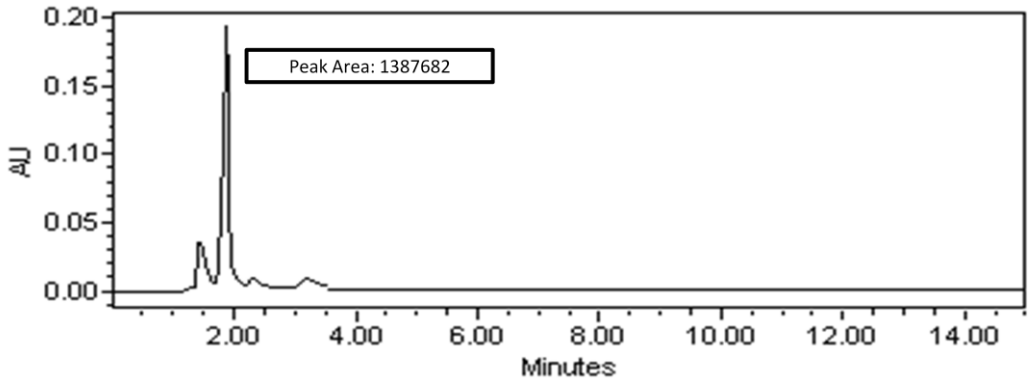
2. Callus



(b)

Salidroside Quantified: 1.054 ± 0.04 mg/g

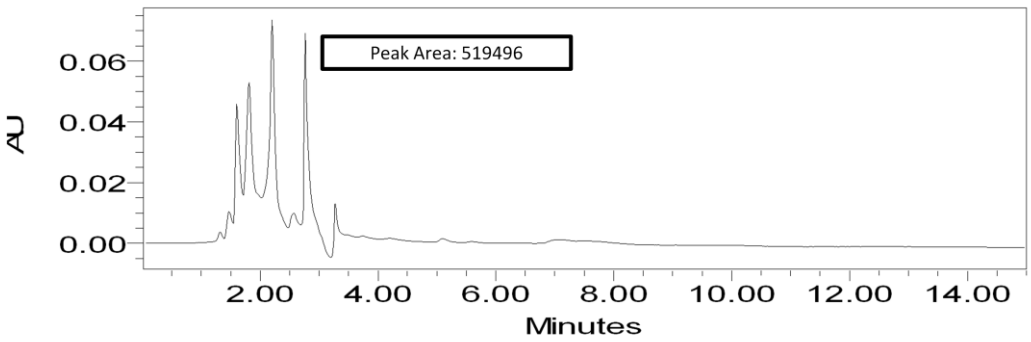
3. Liquid Culture



(c)

Salidroside Quantified: 1.202 ± 0.05 mg/g

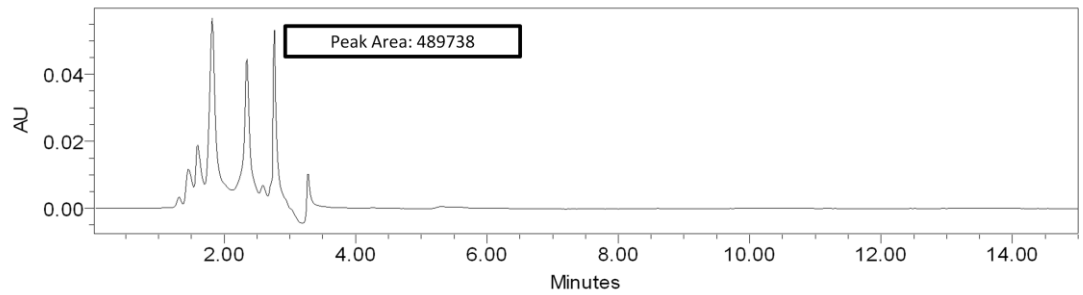
4. Cinnamic Acid



(d)

Salidroside Quantified: 0.450 ± 0.03 mg/g

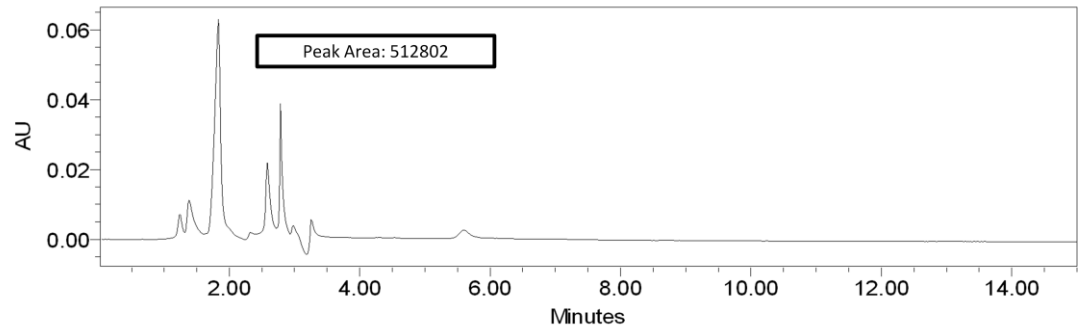
5. Chitin



(e)

Salidroside Quantified: 0.424 ± 0.05 mg/g

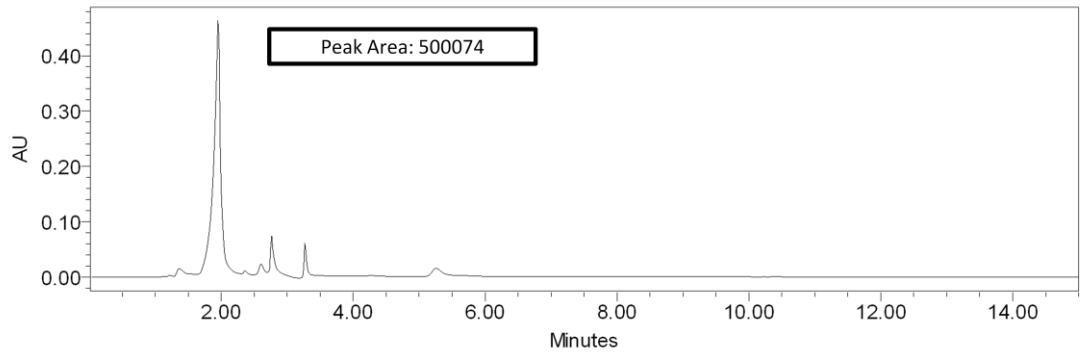
6. L- Phenylalanine



(f)

Salidroside Quantified: 0.444 ± 0.06 mg/g

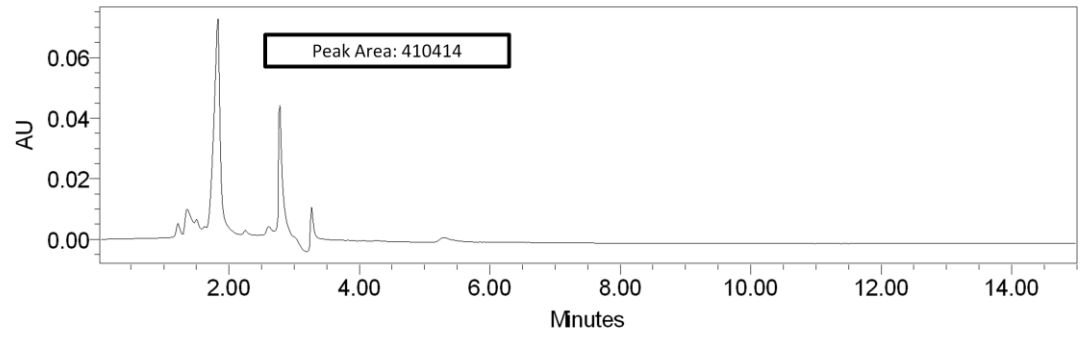
7. Pectin



(g)

Salidroside Quantified: 0.433 ± 0.02 mg/g

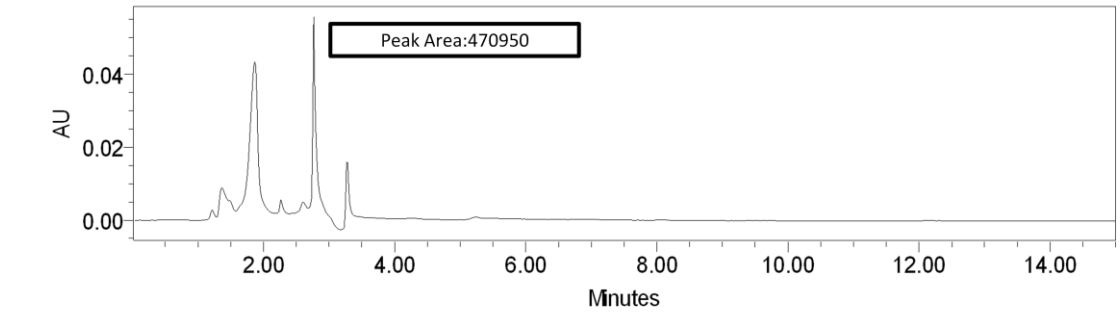
8. Methyl Jasmonate



(h)

Salidroside Quantified: 0.355 ± 0.03 mg/g

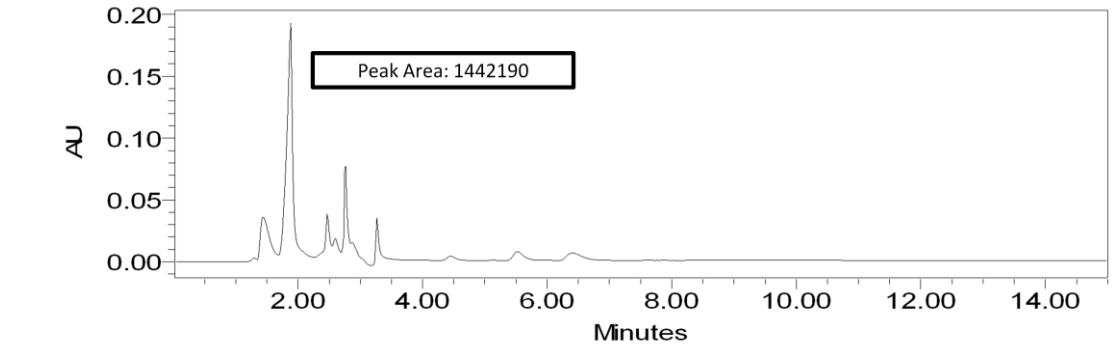
9. Yeast Extract



(i)

Salidroside Quantified: 0.408 ± 0.04 mg/g

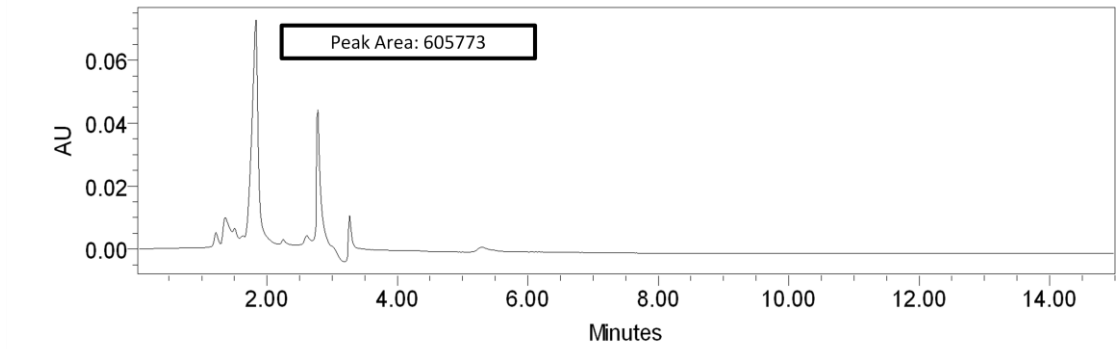
10. Electric Shock



(j)

Salidroside Quantified: 1.249 ± 0.09 mg/g

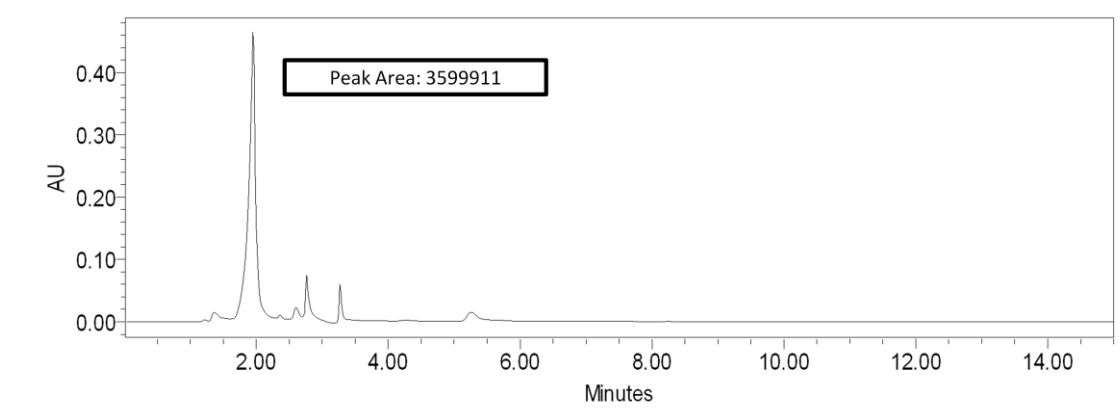
11. Photosynthetic Light



(k)

Salidroside Quantified: 0.420 ± 0.05 mg/g

12. Ultraviolet Light



(l)

Salidroside Quantified: 3.117 ± 0.08 mg/g

Figure 4.17: Chromatogram of (a)Shoot (b)Callus (c)Liquid Callus (d)Cinnamic Acid (e)Chitin (f)L-Phenylalanine (g)Pectin (h)Methyl Jasmonate (i)Yeast Extract (j)Electric Shock (k)Photosynthetic Light (l)Ultraviolet Light

Table 4.6: Salidroside quantified from different tissues of *R. imbricata*

S. No	Sample	Sample Type	Salidroside Quantified (mg/g)
1	Shoot	Shoot	3.687 ± 0.07
2	Callus	Callus	1.054 ± 0.04
3	Liquid Culture	Callus	1.202 ± 0.05
4	Cinnamic Acid	Shoot + Callus	0.454 ± 0.03
5	Chitin	Shoot + Callus	0.424 ± 0.05
6	L- Phenylalanine	Shoot	0.444 ± 0.06
7	Pectin	Shoot + Callus	0.433 ± 0.02
8	Methyl Jasmonate	Shoot + Callus	0.355 ± 0.03
9	Yeast Extract	Shoot	0.408 ± 0.04
10	Electric Shock	Callus	1.249 ± 0.09
11	Photosynthetic Light	Shoot + Callus	0.420 ± 0.05
12	Ultraviolet Light	Shoot	3.117 ± 0.08

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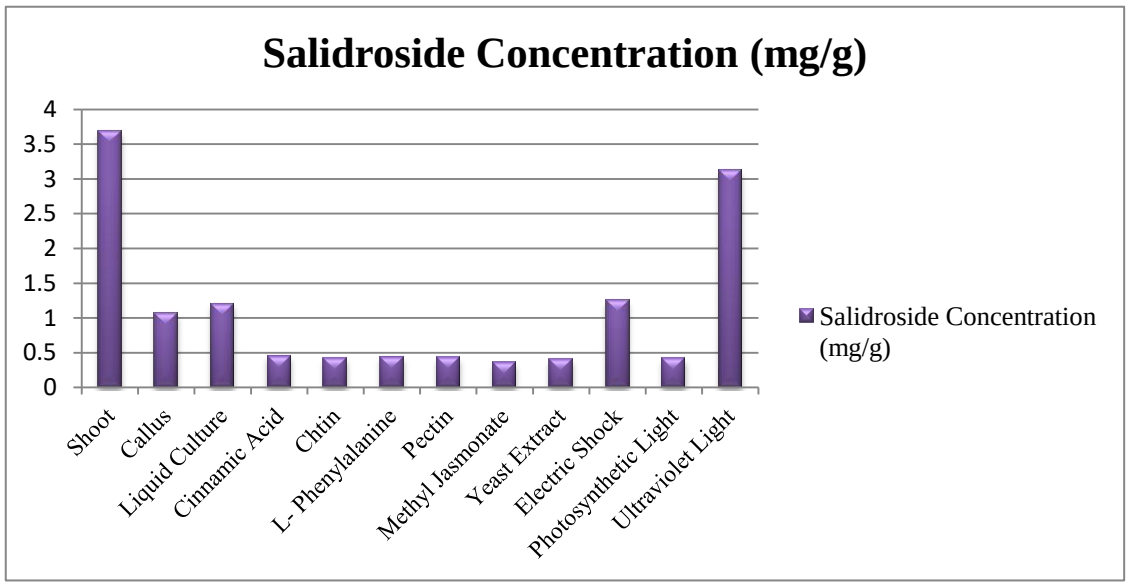

Graph 4.1: Comparitive graphical representation of quantifies salideoside amount from different tissues of *R. imbricata.*

From the above quantification data, the use of elicitors did not provide significant elicitation in salidroside content whereas growth and development parameters were enhanced signifiantly in photosynthetic light by using different combination of growth hormones Optimized liquid medium also provide an alternative mode for growth and development of plantlets which could be further optimized for marker compound production.

DISCUSSION

The cultivation of medicinally important Tran- Himalayan plant, *R. imbricata* is very difficult in its natural environment. There are many problems encountered in mass propagation of this plant. The employment of labour is one of the most difficult problem due to expensive availability at high altitudes severe damage to the health of the workers working under these extreme climatic conditions. Even if feasible, the destruction caused to the vulnerable ecosystem would be so drastic that it will simply add the plant varieties to Red list.

Thus, a better way to approach this issue would be the use of biological engineering technologies, such as plant tissue culture and metabolic engineering to obtain medicinally important biological components synthesized by the human intervention. And this the basic idea behind doing this project.

The rationale behind this study was to develop an *in vitro* system for micropropagation of *R. imbricata* and using this system for enhanced biosynthesis of salidroside using different elicitors. The best medium for callus regeneration was found to be MS medium supplemented with TDZ (1mg/L). Depending upon the endogenous concentration of growth hormones applied to different explants under same in vitro conditions, different responses were noted with respect to plant regeneration [97]. The best growth was reported in MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l), which is in accordance with results reported by S. Sharma *et al (2016)* [31]. According to the results obtained in the elicitations, the significant growth enhancement was seen in plants of *R. imbricata* grown in photosynthetic light. The increased plant height, stem girth and multiple shooting was reported for the first time as the effect of being grown in photosynthetic light. The earlier studies find the mention of increased growth and development rate but no morphological changes [91]. As for electricity,

there was 3 fold increase in the leaf size, which is similar to the findings of Evans Kaimoyo. The results also supported the enhancement of salidroside but not as high as reported earlier [94].

The in vitro grown shoots of *R. imbricata* gave significantly high content of salidroside as compared to salidroside content in field grown root extract. According to the study done by Sahil kapoor *et al* [98], the salidroside content present in field grown parts was 1.08mg/g whereas the amount of salidroside present in *R. imbricata* tissue cultured shoots grown in MS media supplemented with BAP (1 mg/l) + IBA (2 mg/l) was 3.6 mg/g, which is nearly 3.4 folds higher. This finding as it is solves the basic purpose of our study.

In addition to this, the elicited plants also showed enhanced salidroside amount where callus elicited with electric shock gave a positive response. The amount achieved was greater than control callus grown in optimized MS media. But the results achieved were not as impressive as reported by Evans Kaimoyo *et al* in *Pisum sativum* [94]. When the salidroside content of callus cultures grown in Liquid MS media supplemented with similar growth hormones as in optimized solid MS media, slight enhancement was observed. Despite the same media composition, the increased metabolite concentration holds a strong ground for up scaling the research. The similar findings were also reported in suspension cultures of *Rhodiola sachalinensis* by Jay Xu [99]. UV also proved to be an efficient elicitor in enhancing the concentration of salidroside which is in accordance with the research done by Y. K. Bernard (reference) [93]**.** In addition to this, the liquid culture optimization to enrich the biomass of the plant was successfully done with 5.35 fold increase in the callus in comparison to callus grown on solid media. The similar findings were also reported by D. Popli *et al.* (2016) [100].

So, the present findings provide platform to upscale the study to bioreactor level. Thus help to achieve desirable plant growth and marker production can be carried out at commercial scale in order to meet the demands of pharmaceutical industries.

CHAPTER 5

CONCLUSION

CHAPTER 5

CONCLUSION

The growth of *Rhodiola* plants is very slow and these are not found in great profusion in the wild because of the extreme environments where they are grown. Secondly, extraordinarily high demand is made on these resources due to their high medicinal and pharmaceutical value. Owing to this huge demand, the *Rhodiola* plants are inadequately exploited. This problem could be solved to a certain extent by application of technologies such as plant tissue culture where seedlings could be obtained under "near natural" environmental conditions.

Though having vast potential in terms of pharmaceutical values, not enough work has been done to save the plant in its native place. Through this project, we have optimized the liquid medium for improving the growth and development of callus. It has enhanced by 5.35 folds within 20-25 days as compared to controls grown in solid media. Moreover, we have used 12 different elicitors for enhancing the concentration of marker compound that is salidroside under in vitro conditions but photosynthetic light and UV gave some significant elicitation along with good growth and development, whereas some morphological changes were observed in cultures grown in electric shock as an elicitor.

Hence, the current study possesses the robust potential in large scale propagation of this plant and its secondary metabolite production. It also provides a platform for up scaling this research to bioreactor level so that desirable plant growth and marker compound production can be carried out at commercial scale. Combining together these technologies can provide us with high rates of required metabolites. This will not only ensure easy availability of these medicinally important metabolites in pharmaceutical industries but also help to lower down the cost of varied medicines, thus benefitting the masses on the whole.

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List of Publications:

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Abstract Publication

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