MOLECULAR CHARACTERIZATION OF *RHODIOLA IMBRICATA* FOR BIOSYNTHESIS OF SALIDROSIDE AND ROSAVIN

ENROLLMENT NO.   123820

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CERTIFICATE FROM SUPERVISOR

This is to certify that the work entitled "**Molecular Characterization of *Rhodiola imbricata* for Biosynthesis of Salidroside and Rosavin**" submitted by "**Shubham Sharma**" in partial fulfillment for the award of degree of "5 Year Dual Degree Programme B.Tech - M.Tech in Biotechnology" at Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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DECLARATION

I certify that

- The work contained in this thesis is original and has been carried out by me under the guidance of my supervisor.
- The work has not been submitted to any other organization for any degree or diploma.
- Whenever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

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SUMMARY

Rhodiola imbricata EDGEW., a perennial herb of the family Crassulaceae is found in extreme regions of Himalayas at an altitude of 4000–5000 m. It is also available in border regions of Leh – Ladakh, China and Tibet. In traditional folk medicines of China, Tibet, Mongolia and the former Soviet Republics, *R. imbricata* has been used widely for its medicinal properties in order to increase work productivity, physical endurance, longevity and to treat asthma, fatigue, impotence, haemorrhage and gastrointestinal ailments. It also possess various pharmacological activities like hepatoprotective, radioprotective, cytoprotective, wound healing, Immunomodulatory, anti-fatigue, neuroprotective, anticancerous etc., which is due to the presence of various phytochemicals such as p-tyrosol, Salidroside, Rosavin, gallic acid, etc. To the best of our knowledge, this is the first report on molecular characterization of *Rhodiola imbricata* for biosynthesis of Salidroside and Rosavin. In this study, HPLC conditions for the quantification of Salidroside and Rosavin in in vitro grown callus, shoots and roots maintained at $15 \pm 2°C$ were optimized. HPLC analysis revealed that in callus, in vitro shoots and in vitro roots, Salidroside was found to be as 2.401 ± 0.05^f µg/mg, 2.875 ± 0.08^d µg/mg and 0.712 ± 0.04^d µg/mg of fresh weight respectively while no traces of Rosavin was found in these tissues. Contrasting conditions were also developed using MS media & different growth hormones combinations and different temperatures for the accumulation of secondary metabolites and for transcriptome generation. It was found that in callus maintained at $15 \pm 2°C$, MS medium containing BAP (1 mg/l) + IBA (2 mg/l) resulted in 2.401 ± 0.05^f µg/mg of Salidroside, while in in vitro shoots maintained at $15 \pm 2°C$, MS medium containing BAP (1 mg/l) + KN (2 mg/l) resulted in 2.875 ± 0.08^d µg/mg of Salidroside. Also in in vitro roots of plantlets maintained at $15 \pm 2°C$, MS medium having BAP (2 mg/l) + IBA (4 mg/l) resulted in 0.712 ± 0.04^d µg/mg of Salidroside. It was also found that callus growth, shoot multiplication and rooting were observed earlier at $15 \pm 2°C$ as compared to other temperatures including $10 \pm 2°C$, $25 \pm 2°C$ and $30 \pm 2°C$ along with higher Salidroside accumulation. Genes responsible for the biosynthesis of Salidroside and Rosavin in callus and in vitro shoots were identified and the sequences were also generated for those genes. Therefore, this study will provide a platform for further molecular exploration of medicinally important secondary metabolites in *R. imbricata*.

Signature of Student   Signature of Supervisor

Name - Shubham Sharma   Name - Dr. Hemant Sood

Date -   Date -

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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL FORM
GSH	Glutathione
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
HPLC	High Performance Liquid Chromatography
Tert-BHP	Tert-butyl hydroperoxide
ROS	Reactive oxygen Species
TNF-α	Tumor necrosis factor-α
IL-6	Interleukin 6
PBMC	Peripheral blood mononuclear cell
NF-κβ	Nuclear factor-κβ
LPS	Lipopolysaccharides
LD_{50}	Lethal Dose, 50%
HT-29	Human colon adenocarcinoma cells-29
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
RAE	*Rhodiola* aqueous extract
NK	Natural killer
PARP1	Poly (ADP-Ribose) Polymerase 1
TGase	Transglutaminase
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase
TyrDC	Tyrosine decarboxylase
RcTyrDC	Recombinant Tyrosine decarboxylase
MS	Murashige and Skoog
4-HPAA	4-Hydroxyphenylacetaldehyde
Phe	Phenylalanine
Tyr	Tyrosine
PAL	Phenylalanine ammonialyase
4CL	4-Coumarate:CoA ligase
CCR	Cinnamyl-CoA reductase
CAD	Cinnamyl alcohol dehydrogenase
DRDO	Defence Research and Development Organisation
IBA	Indole-3-butyric acid
KN	Kinetin
BAP	6-Benzylaminopurine
GA_3	Gibberellic acid
TDZ	Thidiazuron
HCl	Hydrochloric acid
NaOH	Sodium hydroxide

CHAPTER 1

INTRODUCTION

The stone crop family, Crassulaceae comprises of over 1400 species which is disseminated in 33 genera including *Rhodiola* (Rose root, Golden root or Arctic root) and is commonly distributed worldwide especially in the Northern Hemisphere and South Africa (Gupta et al., 2007). The descriptions, distribution and collection sites of the various species of the family are available in different literatures (Kumar et al., 2007). The species of this family propagates at an altitude of 2700-5000 m in drained soil and has been acknowledged to have immense medicinal potential from time immemorial (Mishra et al., 2007). The *Rhodiola*, which consists of around 90 species, are widely dispersed in cold desert region of the Northern Hemisphere at high altitude (Yidong Lei, et al., 2003). In traditional medicines of Tibet and other regions, various *Rhodiola* species have been used for the treatment of weakness and long term illness due to infection for over 1000 years (Xiong, 1995; Rohloff, 2002).

In India, six species of *Rhodiola* are there, namely *R. heterodonta, R. imbricata, R. quadrifida, R. sinuate, R. tibetica, and R. wallichiana* (Chaurasia and Gurmet, 2003). Crassulaceae is the plant family to which *Rhodiola imbricata* Edgew. previously known as *Sedum roseum* (Linn.) Scop. belongs. It is a perennial herb which is succulent in nature, locally known as rose root due to the rose- like attar (fragrance) of the fresh cut rootstock (Chaurasia et al., 2007). In India it is commonly known as Rose root, Golden root, Arctic root, Shrolo, Stone crop and/or Himalayan stone crop (Chaurasia et al., 2007; Chaurasia and Singh, 1996). Roots along with rhizomes are the parts of this plant that are generally used. Lately, the root of *R. imbricata* has been receiving a lot of attention. *R. imbricata* is a cold tolerant, medicinal plant with adaptogenic properties.

R. imbricata Edgew. [(Synonyms: *Rhodiola imbricatum Edgew*.; *Sedum imbricatum* Walp.; *Sedum imbricatum* Hook.f. & Thomson; *Sedum rhodiola* auct. Non DC.; *Sedum roseum*); (Common names: Golden root, rose root, arctic root or Shrolo)] is a dioecious plant, native around whole of the Northern hemisphere and originating in mountain regions of South West China (Chaurasia et al., 2007; Chaurasia and Singh, 1996). It is a slow growing, perennial garden plant that extends from the Pakistan, India, Nepal to China. In India, the plant grows wildly primarily on rocky slopes, wet places, higher passes at high altitudes (14000-18500 ft above mean sea level) in the trans-Himalayan cold desert and in high Arctic latitudes and Eurasia mountain regions (Khanum et al., 2005). It is commonly found in Indus and Leh valley of Indian trans-Himalaya (Chaurasia et al., 2007; Chaurasia and Singh, 1996).

R. imbricata (Figure 1) is a succulent herb with a thick rhizome which is golden from outside and pink from inside, 10-35 cm with rose scented massive rootstock; leaves 1.3-3 cm long, oblong to narrow elliptic, nearly entire; flowers pale yellow in congested cluster, surrounded by an involucres of leaves; fruits are 4-5, many seeded. Flowering and fruiting in this plant occurs in July-

September (Chaurasia et al., 2007; Chaurasia and Singh, 1996). Classification of *R. imbricata* is given below.

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Rosales
Family	Crassulaceae
Genus	*Rhodiola*
Species	*imbricata* EDGEW

Figure 1. *R. imbricata* plant growing on rocky slopes of Ladakh region, India (Tayade 2015)

Analysis of different *Rhodiola* species revelaed six groups of active principles in their chemical compositions (Khanum et al., 2005).

- Phenylpropanoids: rosin, rosavin, rosarin. The name rosavin includes these three.
- Phenylethanol derivatives: tyrosol, rhodioloside (salidroside, rhodosin).
- Flavonoids: acetylrhodalgin, rhodionin, rhodiosin, rhodiolin, tricin.

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- Monoterpenes: rosiridol.
- Triterpenes: β-sitosterol, daucosterol.
- Phenol acids: hydroxycinnamic, chlorogenic and gallic acids.
- 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 (Tayade 2015).

In Tibet and other regions, for the treatment of undying weakness and illness due to infection, *Rhodiola* species have been used as traditional medicines for over 1000 years (Xiong, 1995; Rohloff, 2002). *R. imbricata* is an important traditional medicinal plant and is widely used as food crop and is distributed in trans-Himalayan cold desert regions. Roots of *R. imbricata* are used for treatment of cough, cold, lung problems, fever, pulmonary complaints and loss of energy in Tibetian and Amchi system of traditional medicine (Chaurasia et al., 2007). Modern pharmacological studies have showed that *R. imbricata* aqueous, ethanolic and hydro-alcoholic root extracts possess anti-cellular and immunomodulatory potential (Mishra, Ganju, Singh, 2012), immunostimulatory activity (Mishra et al., 2006; Mishra et al., 2009), adjuvant activity (Mishra et al., 2010), adaptogenic activity (Tulsawani et al., 2011; Gupta et al., 2008), radioprotective efficacy (Arora et al., 2005; Goel et al., 2006; Chawla et al., 2010), radiomodulatory (Arora et al., 2008), cytoprotective (Kanupriya et al., 2005), antioxidant potential (Kanupriya et al., 2005), free-radical scavenging activity and metal chelating activity (Arora et al., 2008), cold, hypoxia and restraint (C-H-R) exposure and post-stress recovery (Gupta et al., 2010), cold, hypoxia and restraint (C-H-R) hypothermia induced by stress and recovery after stress: mechanism and action (Gupta et al., 2009), anti- proliferative effects (Mishra et al., 2008), anti-cancerous (Mishra et al., 2008), potential of healing dermal wound (Gupta et al., 2007), hepatoprotective effect (Senthilkumar et al., 2014), radical scavenging and antiproliferative activity of extracts in HT-29 human colon cancer cells (Senthilkumar et al., 2013), and found to be safe (Saggu et al., 2006; Gupta et al., 2008).

In light of the aforementioned ongoing research, following research gaps in *R imbricata* research have been noticed.

- Although, various *in vivo* and *in vitro* pharmacological potentials are well documented in polar extracts, the active principle of the plant in these extracts still remains a myth.
- Moreover, the plant is widely used as an edible plant in India, Nepal, Tibet, and China, its nutritional quality with respect to its vitamins, fatty acids, amino acids, and mineral content still remained unexplored in *R. imbricata* root except the species was tested only for the heavy metals such as As, Pb, Hg, Cd, Zn, Cu, and Cr.

- The volatile, semi-volatile and polar compounds in different extracts need to be analyzed to ascertain the bioactivity and pharmacological potential of the plant.
- Recent ongoing research in the plant revealed vital bioactivity in root/rhizome of the plant tested in different solvents. However, the active principles in these extract still remained unrevealed.

Due to the presence of mentioned pharmacological activities, *R. imbricata* can be used by pharmaceutical industries to produce medicines to increase stamina, work productivity, longevity and to decrease fatigue, stress and depression. Thus taking into consideration, high demand of *Rhodolia* raw material worldwide for pharmacological usage, occurrence of species in harsh arctic or mountainous cold disserts, over exploitation from natural populations, inherent difficulties in cultivation, this study has been proposed to investigate the possibility of production of bioactive compounds Salidroside and Rosavin of this species using biotechnological intervention.

To best of our knowledge, no reports on molecular characterization for secondary metabolite production in *R. imbricata* are available till date. Therefore, the following objectives have been placed under studies.

- Quantification of Salidroside and Rosavin present in different in vitro grown tissues of *R. imbricata*.
- Identification of key genes responsible for biosynthesis of Salidroside and Rosavin in *R. imbricata*.
- Development of contrasting conditions for accumulation of secondary metabolites for transcriptome generation.

CHAPTER - 2

REVIEW OF

LITERATURE

Taxonomy and botany of *R. imbricata*

In developing or underdeveloped countries, around 80% of inhabitants depend mostly on traditional medicine for the needs related to health care. A majority portion of health care need comprises the utilization of plant extracts or the bioactive compounds formed by the plants. Requirement of quality control profiles and standardization for accurate recognition of the concerned species, whether it is in fresh, dried or powdered state, is one of the disparagements of herbal medicine (Tayade, 2015). In preparation and administration of herbal medicine, real threat is the species misclassification and flawed changeover (Tayade, 2015). Most of the herbs which are mistaken for one another are the herbs having extremely comparable appearance to the unexperienced eye. The misclassification of species and the mistaken substitution of herbs have also given rise to serious adverse effects (Tayade, 2015). Therefore, the taxonomic and botanical classification and recognition for the accurate species from its natural habitat of *R. imbricata* mentioned earlier in Introduction is quite useful.

Cultivation and propagation of *Rhodiola* species

For its root yield, *Rhodiola* spp. can be efficaciously grown using methods of organically growing plants in South Finland according to the cultivation experiences (Tayade, 2015). For natural winter stratification, seeds have to be sown in autumn in order to produce seedlings. For around one year before transplantation, these seedlings should be kept in pots due to the slow growth of plants during first 2-3 years. After four years from sowing, the first root yield is harvested. The root weight and root yield intensely depends on age of the plant.

Cultivation and propagation practices indicate that *R. imbricata* plant is propagated through seeds as well as rootstocks cuttings. 65% of seed germination was achieved in field conditions while in case of rootstocks plantation 86% of the survival rate has been achieved. Hence, it was found to be easily propagated through division of rootstocks and 3-4 years plants are ideal for suitable rootstocks plantation. Three-five years old plants are considered ideal for rootstocks plantation (Chaurasia et al., 2007).

Under in vitro conditions, callus induction and indirect shoot regeneration was found to be superlative in MS media having BAP (1 mg/L) and IBA (2 mg/L), while using MS media having cytokinins like BAP (1 mg/L) and KN (2 mg/L) resulted in direct shoot formation and proliferation. MS media having BAP (2 mg/L) and IBA (4 mg/L) resulted in in vitro root induction

within 25 – 30 days. Survival rate of 75 - 80 % was shown by in vitro rooted plantlets after hardening in greenhouse conditions (Sharma et al., 2017).

Tasheva et al. (2010) have developed efficient schemes for the regeneration and micropropagation of *R. rosea*. Monika et al. (2005) showed successful encapsulation of differentiating callus and axillary buds in calcium alginate beads in *R. kirilowii*. These capsules were able to develop into shoots and plantlets on solid MS basal medium. Yan et al. (2012) developed a modified temporary immersion bioreactor with forced ventilation which decreased the hyperhydration rate and improved shoot quality along with their multiplication ratio in *R. crenulata*.

Pharmaceutical products from *Rhodiola* species

Some of the pharmaceutical products including Rosavin ™ (Ameriden), Siberian *Rhodiola rosea* (Supernova Naturals), Perfect *Rhodiola rosea* (Perfect Supplements), Vitango® (Dr.Willmar Schwabe GmbH & Co. KG, Germany), *Rhodiola rosea* Capsules (SP Herbals, Kerala, India) containing recommended 3:1 ratio of Rosavin to Salidroside have been developed from *R. rosea* to act as anti-stress, anti-fatigue and anti-depressants. In pharmacological research, special emphasis has been put on *R. rosea* in the former Soviet Union as Russian researchers, it has been characterized as adaptogen. (Kelly, 2001).

Pharmacological properties of *R. imbricata*

In a study (Senthilkumar et al., 2014), hepatoprotective activity of acetone extract of *R. imbricata* rhizome against liver toxicity induced by paracetamol estimated. To induce hepatic damage, Paracetamol was administered in Wistar rats. As treatment groups, silymarin (25 mg/kg) and rhizome acetone extract of 200 and 400 mg/kg dosage were used. Analysis of blood samples were carried out for hepatic injury biochemical markers. Also, estimation of liver antioxidants were carried out using tissue samples along with histopathological studies. An elevation of glutothione peroxidase (19.26 mg GSH consumed/min/mg protein), superoxide dismutase (0.326 units/min/mg protein), reduced glutathione (16.2 μmole of GSH/mg protein) and catalase (185.03 μmole of H_2O_2 consumed/min/mg protein) were revealed by the analysis of rats (400 mg/kg) treated with extract. Moreover, improvement of the serum biochemical parameters like SGOT, alkaline phosphatase, lipid profile and serum glutamic pyruvic transaminase SGPT were also observed in the groups that were treated as compared to the control. Hepatic cells were significantly protected from damage by the

oral administration of different doses of rhizome acetone extract. In extract treated rats, the haematological and biochemical parameters were also normal as compared to the standard (silymarin) groups and control. The presence of some important phenolic compounds were also revealed by the HPLC analysis, which could be accountable for this hepatoprotective activity. This study showed that *R. imbricata* could be considered as a virtuous natural source of the hepatoprotective agent.

Antioxidant and cytoprotective activity of alcoholic as well as aqueous extracts of rhizome of *R. imbricata* on cytotoxicity induced by tert-butyl hydroperoxide (tert-BHP) in human macrophages (U-937) was also reported (Kanupriya Prasad et al., 2005). In the presence of tert-BHP, there was a significantly increase in cytotoxicity and apoptosis in treated cells as compared to the cells that were kept as control. The cytotoxicity induced by tert-BHP can be marked to heightened the production of reactive oxygen species which in succession is accountable for decrease in reduced glutathione (GSH) levels. Further, a substantial increase in apoptosis and DNA fragmentation along with decrease in mitochondrial potential were seen. Both alcoholic as well as aqueous extracts of *R. imbricata* rhizome were found to hinder apoptosis, production of free radicals, induced by the tert-BHP and to restore the anti-oxidant levels at concentration of 250 mg/ml to that of the control cells. Higher cytoprotective activities were shown by the alcoholic extract of *R. imbricata* than the aqueous extract. These interpretations proposes that the aqueous and alcoholic extracts of *R. imbricata* have noticeable antioxidant and cytoprotective activities.

It was found that the wounds which were treated with ethanolic extract of *R. imbricata* rhizome healed much faster as showed by the reduced time taken for epithelialization along with enhanced rate of wound contraction (Gupta et al., 2007). As demonstrated by increases in protein, DNA, hexosamine and hydroxyproline contents in contrast to a povidone-iodine ointment treated positive control, collagen synthesis and cellular proliferation at the wound site were increased by the extract treatment and the histological examinations also supported the results. Furthermore, an increase in antioxidant along with a decrease in lipid peroxide levels in the granulation tissue were also observed after treatment. The results suggest that a significant wound healing activity is being possessed by ethanolic extract of *R. imbricata* rhizome.

Evaluation of immunomodulatory activity of *R. imbricata* rhizome aqueous extract was also carried out in human peripheral blood mononuclear cells (PBMCs) and mouse macrophage cell line RAW 264.7 (Mishra et al., 2006). tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production in human PBMCs along with RAW 264.7 cell lines were found to be stimulated by the *R. imbricata* extract. Augmented production of nitric oxide paralleled with lipopolysaccharide (LPS) in

RAW 264.7 was also found. At 250 μg/ml *R. imbricata* extract, p-IκB expression was increased in the human PBMCs. Nuclear translocation of NF-κB in human PBMCs was also activated by the aqueous extract of *R. imbricata* (250 μg/ml), which is analogous to the positive stimulant LPS. This study proposed that activation of proinflammatory mediators was most likely done by *R. imbricata* through transcription factor NF-kB and phosphorylated inhibitory kB. It also established the immunostimulatory potential of *R. imbricata* rhizome aqueous extract that can be used for treating the defective functioning of the immune system.

It was found that, with no acute and sub-acute toxicity, adaptogenic activity was possessed by aqueous extract of *R. imbricata* root (Gupta et al., 2008). To examine this, *R. imbricata* root aqueous extract was orally administered at altered doses in rats, 30 min prior to cold (5 °C)–hypoxia (428 mm Hg)–restraint (C–H–R) exposure. The 100 mg/kg body weight was the maximal effective adaptogenic dose of the extract. In sub-acute toxicity studies, single oral dose of 1 g/kg and 2 g/kg of extract daily one time for 14 days as well as maximal effective single oral dose of 100 mg/kg daily one time for 30 days was administered. At the end of each treatment period, biochemical parameters in serum and blood that are related to lipids (triglycerides, cholesterol), kidney function, liver function, and hematological parameters were estimated. In comparison to control animals, no noteworthy changes in any of the examined parameters in animals of treated group were shown in biochemical and hematological analysis. Also, in organ weight/body weight ratios of any organ which was examined in parallel to the rats that were kept as control, no significant change was observed. More than 10 g/kg was observed to be the oral LD_{50} of the extract, which indicated an adequate brim of safety. In the vital organs of treated animals which were studied, no histopathological changes were observed. These results proposes that an effective adaptogenic activity with no acute and sub-acute toxicity is possessed by *R. imbricata* root aqueous extract.

Methanol and acetone extracts of *R. imbricata* logged greater phenolic content and exhibited comparable antioxidant activity with standard reference (Senthilkumar et al., 2013). Additionally, upon treatment at higher concentration (200 μg/mL) (acetone and methanol, 84% and 84%, respectively), proliferation of HT-29 cells was also inhibited. On examination, methanol extract showed both dose dependent as well as time dependent antiproliferative activity, whereas, the acetone extract exhibited inhibitory activity only in a concentration dependent manner.

In K-562 cell line of human erythroleukemic, *R. imbricata* aqueous extract was studied for its anti-proliferative effects using MTT cell proliferation assay (Mishra et al., 2008). After 72 h incubation with *R. imbricata* aqueous extract at 100 and 200 μg/ml, the proliferation of K-562 was significantly decreased. However, in normal human peripheral blood lymphocytes or mouse macrophage cell line RAW-264.7, almost no suppressive effects could be detected. It was found that

reactive oxygen species (ROS) inside the K-562 cells were also induced after overnight incubation with 200 μg/ml of *R. imbricata* aqueous extract. In propidium iodide (PI) and annexinV-FITC staining of K-562 cells which were treated with *R. imbricata* aqueous extract for 72 h, increased ROS generation was observed, which may cause apoptosis. Moreover, in early and late period of exposure, *R. imbricata* aqueous extract detained the progression of cell cycle in G2/M phase. By increased NK cell cytotoxicity, the anti-cancer activity of *R. imbricata* aqueous extract was also confirmed. These observations proposes that the *R. imbricata* rhizome aqueous extract has very effective anti-cancerous activities, which can be used to treat leukemia cancer.

Presence of all these pharmacological activities can be correlated with the presence of biocompounds such as rosavin, rosin, p-tyrosol, salidroside and gallic acid in *R. imbricata*.

Biosynthesis and molecular exploration

- **Salidroside**

Salidroside protects human erythrocytes by its antioxidant activity and caspase-3 inhibition in a dose-dependent fashion (Qian et al., 2012). It also protects hematopoietic stem cells from oxidative stress by activating *PARP1*, a DNA repair enzyme actively involved in cell apoptosis (Li et al., 2014). Results of various clinical trials have revealed that Salidroside possesses various functions such as anti-cold, anti-fatigue, anti-anoxic, anti-virus, anti-microwave radiation, and anti-tumor. It also possesses various medicinal properties such as preventing illness associated with old age, strengthening attention spans, delaying senility and improving work efficiency. Due to its environmental acclimation activity, it plays important roles in healthcare, military, sports and aerospace.

Precursor of Salidroside is a small phenolic molecule, p-tyrosol. The biosynthetic pathway and regulation of p-tyrosol is not completely understood. People have depicted two different sights on biosynthesis of Salidroside. The first embraces that p-coumaric acid precursor resulting mainly from phenylalanine synthesize tyrosol by a decarboxylase. The second states that tyramine synthesized from tyrosine may be the precursor of tyrosol (Zhang et al., 2011) (Figure 2). Glycosylation of tyrosol is believed to be the last step in biosynthesis of Salidroside (Xu et al., 1998a). Though, the competency of this reaction is governed by tyrosol glucosyltransferase (*TGase*) activity. There are a few reports (Yu et al., 2011; Xu et al., 1998b) conversing the activity of *TGase* in the biotransformation process in *Rhodiola* spp. These investigations were concerned mainly with *R. sachalinensis*.
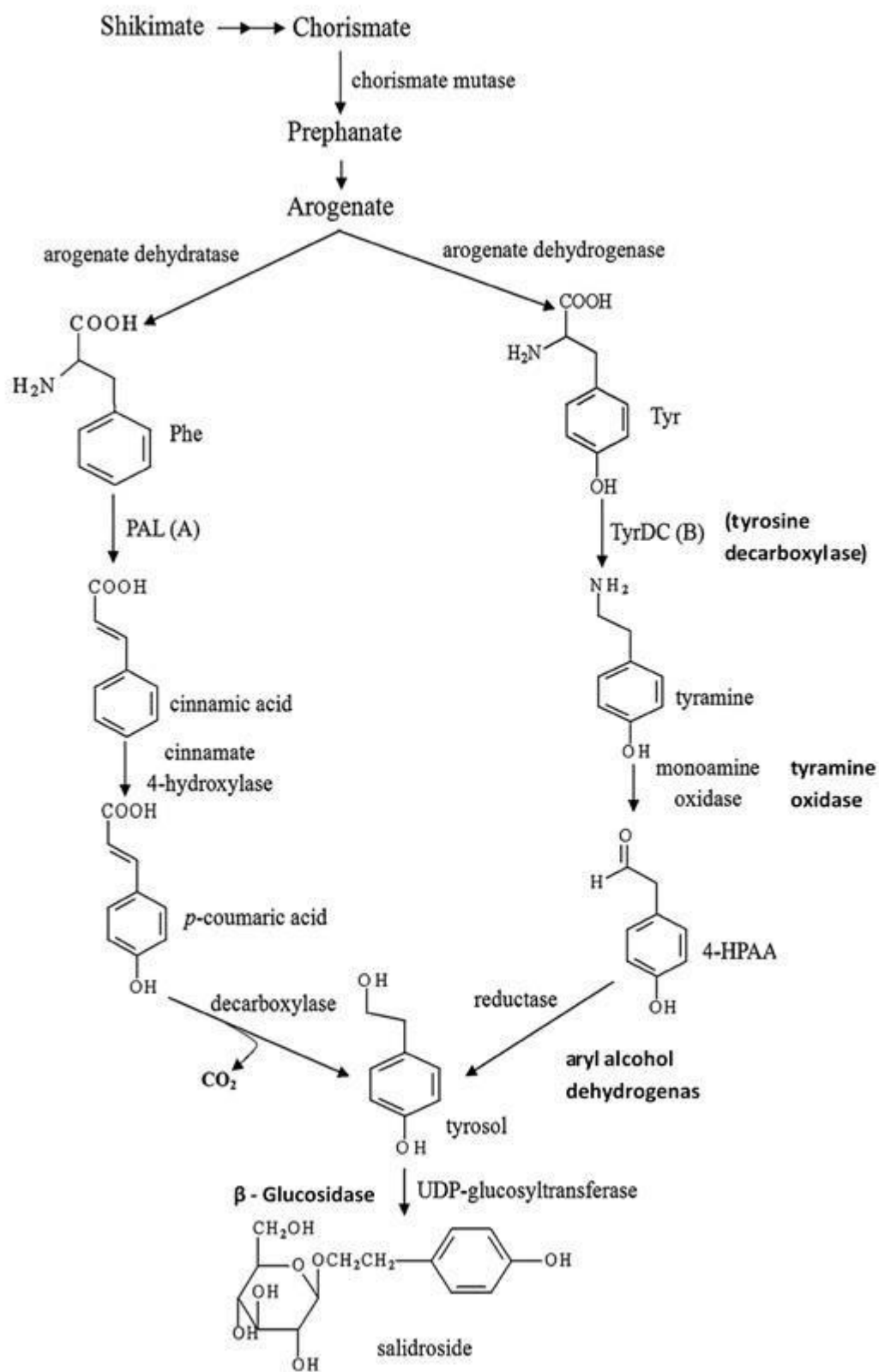
Figure 2. Propounded pathway responsible for biosynthesis of Salidroside (Ma et al. 2008).

Xu et al. (1998b) suggested that due to the lack of harmonisation between tyrosol accumulation and *TGase* activity in the cells and low efficiency of glycosylation, low quantity of Salidroside was observed in *R. sachalinensis* cell culture. Ma et al. (2007) showed that in *R. sachalinensis*, conversion of tyrosol aglycone to Salidroside could be regulated by *UGT73B6*. Increased Salidroside content was achieved by overexpression of the *UGT73B* gene in transformed *R. sachalinensis* plants (2-fold increase over untransformed control) and transformed calli (2.4-fold increase). Two other uridine diphosphate (UDP)-glucosyltransferase cDNAs: *UGT72B14* and *UGT74R1* were isolated by Yu et al. (2011). In roots, the first transcript was more abundant while in calli of *R. sachalinensis*, the second was highly expressed. The highest Salidroside production in vitro and in vivo was given by *UGT72B14*. Transformed root cultures of *R. sachalinensis* have also been studied as a probable source of Salidroside. Establishment of transformed root cultures were accomplished by transformation with *Agrobacterium rhizogenes* and liquid MS medium was used for cultivation. Increased biomass and Salidroside accumulation was achieved by medium supplementation with tyrosol (Zhou et al., 2007).

A putative TyrDC gene was isolated from *R. rosea* and the expression level of gene was consistent with the accumulation of Salidroside (György et al., 2009). TyrDC has a key role in tyramine-derived compounds as the overexpression of parsley TyrDC in potato lead to enhanced tyramine-derived compounds synthesis in potato (Landtag et al., 2002). In another study, TyrDC encoding full-length cDNA was cloned and functionally identified by feeding intermediate to Tibet-specific *R. crenulata* recombinant protein. With the treatment of different elicitors, analysis of gene expression profiles in hairy root cultures and different tissues were done. The accumulation pattern of Salidroside was also investigated and finally a pathway responsible for biosynthesis of Salidroside in *R. crenulata* hairy root cultures was engineered using metabolic engineering strategy of overexpressing RcTyrDC based on the discoveries above, that enhanced the tyramine, tyrosol and Salidroside biosynthesis (Lan et al., 2013).

Through functional analysis, it was shown that tyrosine was the best substrate of TyrDC. A marked increase in tyrosol and Salidroside content in *R. sachalinensis* was accomplished by overexpression of sense TyrDC, which suggested that TyrDC can regulate the biosynthesis of tyrosol and Salidroside, and thus, TyrDC is most likely to have a crucial function in the first reaction of the pathway responsible for the biosynthesis of Salidroside in *R. sachalinensis* (Ji-Xing Zhang et al. 2011).

In secondary metabolism of plants, UDP-glucosyltransferase catalyzes the synthesis of lower molecular weight glucosides. The final step of Salidroside biosynthesis is a reaction of adding a glucose molecule to tyrosol molecule. It is possible that the UDP-glucose transferase is the enzyme

which is catalyzing the final reaction by using UDPG (an energy-rich and active form of glucose in living organisms) as the glucose donor and tyrosol as its receptor (Shi Ling-ling et al. 2007).

β-D-Glucosidase may possess a dual nature in terms of metabolism of Salidroside. It may participate in both anabolism as well as catabolism reaction. During anabolism reaction, it transfers a glucosyl to tyrosol (the aglycon) and forms Salidroside, while during catabolism reaction, it breakdown Salidroside to glucose and tyrosol. At present stage, it is not clear whether the hydrolase or the transferase activity of the enzyme has priority in in vivo tissues, or whether it acts differently at diverse developmental stages and/or different tissues and plants (Shi Ling-ling et al. 2007).

- **Rosavin**

Cinnamoylglycosides like Rosavin are the phenylpropanoids typically found in *Rhodiola* spp. These were first isolated, identified and designated by Zapesochnaya and Kurkin (1982). Rosavin is recognised to stimulate the spontaneous motor activity (Zapesochnaya et al., 1995), antistress and adaptogenic agent (Kurkin et al., 2007; Sokolov et al., 1990). Its potential antidepressant (Panossian et al., 2005; Kurkin et al., 2006), nootropic (Kurkin et al., 2007), UV-protective (Goldstein et al., 2008) properties were reported. Also, Rosavin in the highest dose significantly decreased the neovascular reaction that were induced in the skin of Balb/c mice after the grafting of L-1 sarcoma cells (Bany et al., 2008).

Both Salidroside as well as the cinnamyl alcohol glycosides are the products of phenylpropanoid metabolism which is derived from phenylalanine. Phenylalanine is a derivative of the shikimic-chorismic acid pathway and Phenylalanine ammonia lyase (*PAL*) is the enzyme that directs the carbon atoms towards the phenylpropanoid metabolites biosynthesis. *PAL* is one of the most widely studied enzyme in the pathway of phenylpropanoid. It transforms phenylalanine to cinnamic acid. At this step, the pathway leaves the main biosynthesis of phenylpropanoid, which would lead to flavonoids, coumarins, or lignans and lignins. Nevertheless, same type of enzymes participates in further biosynthesis of cinnamyl alcohol glycosides. Cinnamyl CoA ester is synthesized from cinnamic acid by hydroxycinnamate:CoA ligase (*4CL*). Cinnamyl-CoA reductase (*CCR*) reduces this CoA ester to cinnamaldehyde. Cinnamyl alcohol dehydrogenase (*CAD*) further reduces the cinnamaldehyde to cinnamyl alcohol. Identification of enzymes that participates in the synthesis of cinnamyl alcohol glycosides has not yet been accomplished. By one glucose transfer, Rosin, which is the simplest glycoside of roseroot is formed. Rosavin is formed from rosin by the addition of an arabinose and rosarin is formed from rosin by the addition of an arabinofuranose

(Figure 3). Based on the type of sugar and the site to which it is connected, additional glycosides may be formed (Grech-Baran et al., 2015).
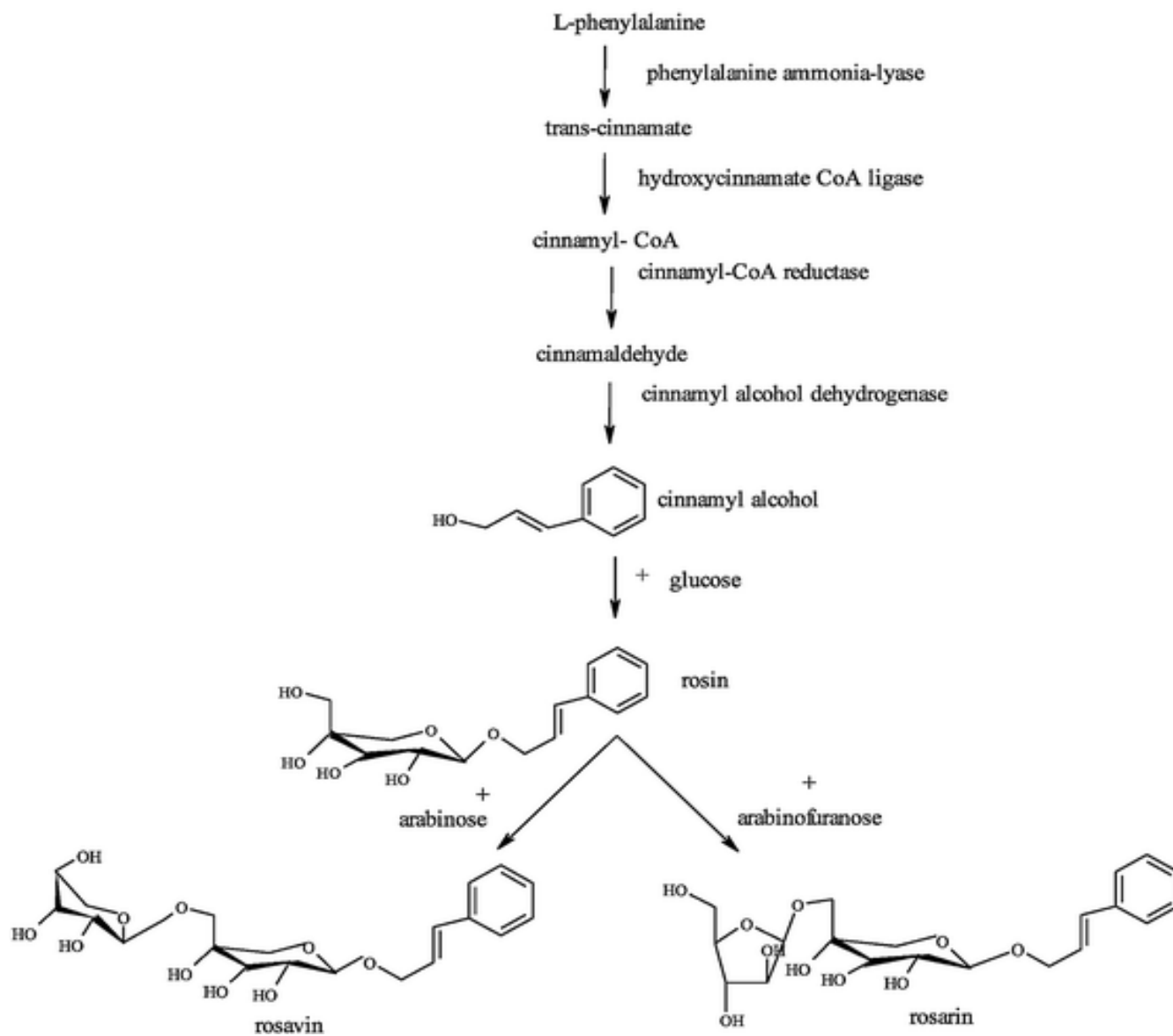
Figure 3. Pathway responsible for the biosynthesis of Rosin, Rosavin and Rosarin (Grech-Baran et al., 2015).

CHAPTER - 3

MATERIALS

AND METHODS

Quantification of Salidroside and Rosavin present in different in vitro grown tissues of *Rhodiola imbricata*

Selection of plant material

The *R. imbricata* tissues were procured from in vitro grown plantlets maintained in plant tissue culture chambers at $15 \pm 2°C$ having 70 % relative humidity, 16 h day/8 h night photoperiod at 40 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density provided by cool white fluorescent tubes (Philips, India) at Jaypee University of Information Technology, Waknaghat, H.P., India (Figure 4).
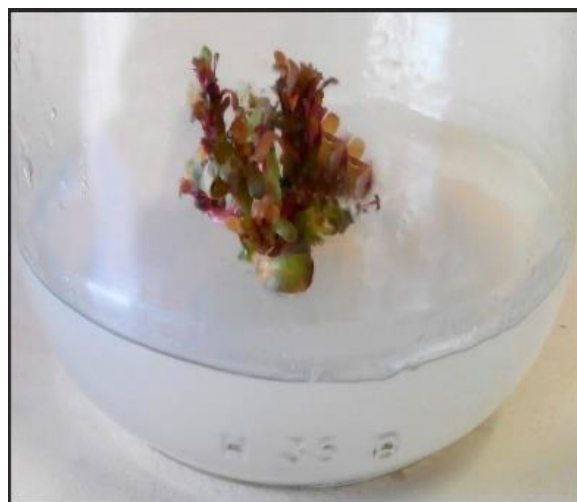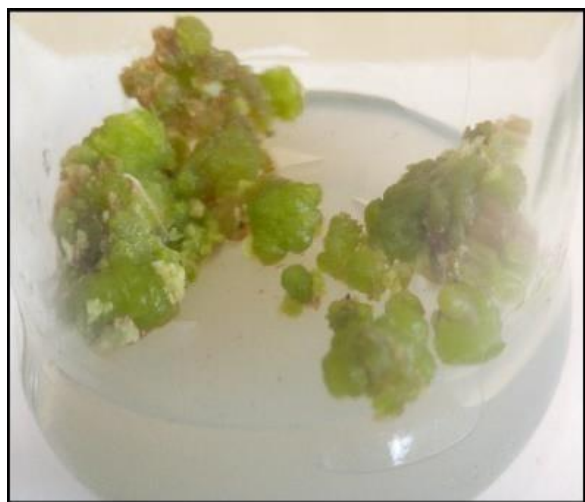
Figure 4. *R. imbricata* in vitro grown tissue cultures in plant tissue culture chambers of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India.

Standard preparation

Standard stock solution was prepared by dissolving 1 mg of each Salidroside and Rosavin standard in 1 ml of 80% Methanol. These stock solutions were diluted 2 – fold to make standard working solutions of 50 ppm and were stored in HPLC vial at 4˚C.

Sample preparation

Fresh tissues from in vitro grown callus (green), shoots and roots were grounded in liquid nitrogen. Approximately 100 mg of grounded tissues from each explant were taken and percolated with 10 ml of 80% Methanol. Sonicator water bath was used to incubate the mixture at 60˚C for 1 hr and was kept at room temperature for overnight. The mixture was filtered through 0.2 µm filter apparatus and the obtained extract was stored in HPLC vials at 4˚C.

Chromatography conditions

Analysis were carried out using a LaChrom MERCK Hitachi system equipped with a L-7400 UV detector, L-7100 dual pump, a vacuum degasser, a manual injection port, L-7350 column oven and a Purospher RP-C18 (3.9x150mm, 5µm particle size) column. The column oven temperature was fixed at 30˚C. Different mobile phase compositions (methanol, acetonitrile and aqueous ammonium acetate of different pH values) with different flow rates were tested to resolve Salidroside and Rosavin standard mixtures. The diode array detector was used to detect Salidroside (at 280 nm) and Rosavin (at 254 nm) while the injection volume was kept 20 µl. The data on Peak area and retention time of Salidroside and Rosavin were recorded. These experiments were performed in triplicates and repeated thrice. The concentration (µg/mg) of the compounds were 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

Development of contrasting conditions for accumulation of secondary metabolites for transcriptome generation

Optimization of MS media and growth hormones for accumulation of secondary metabolites in callus and organogenic cultures

Various MS media (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of IBA, KN, BAP, GA_3 and TDZ (Table 1) with sucrose 3% (w/v) were prepared. HCl (0.1 N) and NaOH (0.1 N) was used to adjust the pH of each media to 5.7 and finally, as a gelling agent, 0.8% (w/v) agar-agar was added. All the media were autoclaved at 121°C and 15 lb/in pressure for 15 - 20 min. Different explants such as leaf, intermodal segments, shoot apex and roots were taken from field grown plant of *R. imbricata* to grow callus, in vitro shoots and roots. These explants were washed under running tap water by using few drops of Labolene and were surface sterilized by using fungicide Bavistin (0.5%) and mercuric chloride (0.1%) followed by 4 - 5 washings in autoclaved water under aseptic conditions. These sterilized explants were cultured on different media combinations (Table 1) and were incubated in plant tissue culture chambers maintained at 15 ± 2°C having 70 % relative humidity, 16 h day/8 h night photoperiod at photosynthetic photon flux density of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India). After 2 months, these cultures were used to prepare samples for HPLC quantification as discussed in "Sample preparation for HPLC" and were quantified as stated previously. The data on tissue used, media, and quantified amount of Salidroside and Rosavin were recorded. These experiments were performed in triplicates and repeated thrice.

Table 1. MS media supplement with different combinations of growth hormones.

S.No.	Medium Name	MS media composition
1.	RI0	MS
2.	RI1	MS + BAP (1 mg/l) + KN (2 mg/l)
3.	RI2	MS + BAP (1 mg/l) + TDZ (2 mg/l)
4.	RI3	MS + BAP (1 mg/l) + IBA (2 mg/l)
5.	RI4	MS + BAP (1 mg/l) + KN (1 mg/l) + IBA (0.5 mg/l)
6.	RI5	MS + BAP (1 mg/l) + IBA (2 mg/l) + GA$_3$ (2 mg/l)
7.	RI6	MS + BAP (2 mg/l) + IBA (4 mg/l)

8.	RI7	MS + IBA (1 mg/l) + KN (3 mg/l)
9.	RI8	MS + BAP (2 mg/l) + IBA (1 mg/l)
10.	RI9	MS + BAP (2 mg/l) + KN (1 mg/l) + GA_3 (2 mg/l)

Optimization of different temperature conditions for accumulation of secondary metabolites

Above mentioned media (Table 1) were used to grow callus, in vitro shoots and roots in four different temperature conditions viz. 10 ± 2˚C, 15 ± 2˚C, 25 ± 2˚C and 30 ± 2˚C. After 2 months, these cultures were used to prepare samples for HPLC quantification as discussed in "Sample preparation for HPLC" and were quantified as stated previously. The data collected for tissue growth, effect of media, growth hormones and effect of temperature on tissue growth and metabolite production were recorded. These experiments were performed in triplicates and repeated thrice.

Sample preparation for Transcriptome analysis

The tissues grown under all the mentioned contrasting in vitro conditions for the production of Salidroside and Rosavin were chosen for transcriptome data generation. Qiagen RNeasy Plant Mini Kit (Qiagen GmbH, Germany) was used to isolate total RNA from *R. imbricata* in vitro grown tissues following manufacturer's instructions. The quality of RNA was assessed on 1% denaturing agarose gel electrophoresis and quantity of RNA was estimated using Nano drop accessory of Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific). RNA extracted from the selected in vitro grown tissues were stored at -80 ˚C until further use.

Identification of key genes for Salidroside and Rosavin biosynthesis

RNA Isolation from *R. imbricata*

Approx. 100 mg of *R. imbricata* shoots and callus (green) each from in vitro grown plantlets maintained at $15 \pm 2°C$ were grounded in liquid nitrogen using mortar & pestle. RNA isolation was carried out using Qiagen RNeasy Plant Mini Kit (Qiagen GmH, Germany) following the manufacturer's instructions. Isolated RNA was then stored at -80 C. Obtained RNA was run on 1% agarose gel in 1X TAE buffer and the presence of bands were checked in Gel documentation apparatus. Concentration of RNA were checked at A_{260} using Nano drop accessory of Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific). Other parameters such as $A_{260/280}$ and $A_{260/230}$ were also checked.

cDNA Synthesis and check

cDNA were synthesized using VERSO cDNA Synthesis kit (Thermo Fischer Scientific) and isolated RNA as a template following the manufacturer's instructions. Synthesized cDNA was then stored at -20 C. Intact cDNA synthesis was checked by PCR amplification using 26s rRNA primers. Amplified products were run on 1% agarose gel in 1X TAE buffer and the presence of bands were checked in Gel documentation apparatus. Concentration of cDNA were checked at A_{260} using Nano drop accessory of Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific). Other parameters such as $A_{260/280}$ and $A_{260/230}$ were also checked. cDNA with higher concentrations were diluted to 150ng/µl.
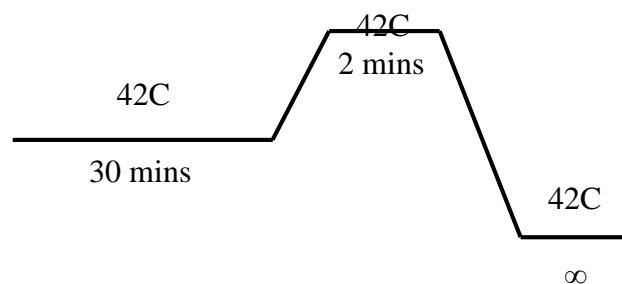
Reaction for cDNA Synthesis –
- 5X cDNA Buffer – 4 µl
- dNTPs – 2 µl
- Oligo dT – 1 µl
- RT Enhancer – 1 µl
- VERSO Enzyme – 1 µl
- RNA – 1 µl
- Nuclease-free Water – 10 µl

Reaction Conditions for cDNA Synthesis –

Reaction for cDNA Check –
- PCR Buffer – 1.25 μl
- dNTPs – 0.25 μl
- Forward Primer – 0.5 μl
- Reverse Primer – 0.5 μl
- cDNA – 1 μl
- *Taq* Polymerase – 0.125 μl
- Nuclease-free Water – 8.875 μl

Reaction Conditions for cDNA Check –
- Initial Denaturation – 94C for 3 mins.
- Denaturation – 94C for 30 secs.
- Annealing – 58C for 45 secs.
- Extension – 72C for 45 secs.
- Final Extension – 72C for 7 mins.

Primers Designing for Salidroside biosynthesis genes

Coding sequences (CDS) of different species were retrieved from NCBI database for TyrDC, UDPGT, β-GD and AAD genes. The sequences retrieved were aligned through multiple sequence alignment using DNASTAR MegAlign and a consensus sequence was obtained for each gene. The portion of consensus sequence having minimum gaps was used to design degenerative primers (Table 2) for each gene using Primer 3 software.

Table 2. IUPAC system for degenerative nucleotide nomenclature.

Single-letter Code	Nucleotide's	Explanation
A	A	**A**denine
C	C	**C**ytosine
G	G	**G**uanine
T	T	**T**hymine
1	I	**I**nosine
R	A or G	pu**R**ine
Y	C or T	p**Y**rimidine
M	A or C	a**M**ino
K	G or T	**K**eto
S	C or G	**S**trong interaction
W	A or T	**W**eak interaction
H	A or C or T	not G, **H** follows G in alphabet
B	C or G or T	not A, **B** follows A in alphabet
V	A or C or G	not T/U, **V** follows U in alphabet
D	A or G or T	not C, **D** follows C in alphabet
N	A or C or G or T	a**N**y

Primers Designing for Rosavin biosynthesis genes

Coding sequences (CDS) of different species were retrieved from NCBI database for PAL, 4CL, CCR and CAD genes. The sequences retrieved were aligned through multiple sequence alignment using DNASTAR MegAlign and a consensus sequence was obtained for each gene. The portion of consensus sequence having minimum gaps was used to design degenerative primers (Table 2) for each gene using Primer 3 software.

PCR Amplification

PCR reaction mixtures of 12.5 µl containing PCR buffer, dNTPs, forward and reverse primers of respective genes, cDNA (*R.imbricata*), *Taq* polymerase and nuclease free water were optimized.

Gradient PCR was set at different temperatures (T_m) for 40 cycles to optimize the T_m for each primer at given reaction conditions. After completion of reaction, the samples were run on 1.2% agarose gel to check for the desired amplification.

Reaction –

- PCR Buffer – 1.25 μl
- dNTPs – 0.25 μl
- Forward Primer – 0.3 μl
- Reverse Primer – 0.3 μl
- cDNA – 1 μl
- *Taq* Polymerase – 0.2 μl
- Nuclease-free Water – 9.2 μl

Reaction Conditions –

- Initial Denaturation – 94C for 3 mins.
- Denaturation – 94C for 30 secs.
- Annealing – T_m for 45 secs.
- Extension – 72C for 1 min.
- Final Extension – 72C for 7 mins.

Gel Extraction and purification

After amplification, bands of desired sizes were excised from the gel under Medox UV Transilluminator in case of multiple banding and were purified using Qiagen QIA Quick Gel Extraction kit (Qiagen GmbH, Germany) following the manufacturer's instructions. Purified samples were run on 1.2% agarose gel in 1X TAE buffer to confirm the desired excision. Concentration of purified products were checked at A_{260} using Nano drop accessory of Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).

Sequencing

After Purification, 30 μl of purified/unpurified products along with 10 μl (10 μM) of primers of respective genes were sent to Xcelris Labs Limited, Ahmedabad, Gujrat, India for Sanger sequencing of the amplified products by ABI 3730xl 96 capillary system using Big Dye Terminator v3.1 kit.

CHAPTER - 4

RESULTS AND

DISCUSSION

<u>**Quantification of Salidroside and Rosavin present in different in vitro grown tissues of _Rhodiola imbricata_**</u>

Optimization of HPLC conditions

Different mobile phase compositions (methanol, aqueous ammonium acetate and acetonitrile of different pH values) were tested to resolve Salidroside and Rosavin standard mixtures. The result was that the optimum resolution could be achieved using 0.16 M ammonium acetate buffer (B), adjusted to pH 5.6 and 100% methanol (A) as mobile phase with isocratic elution: 66A/34B for Salidroside and 60A/40B for Rosavin in 15 min with flow rate of 1.0 mL min^{-1}. Salidroside and Rosavin standards were quantified with peak area of 172320.5 ± 0.09^b and 1830682.5 ± 0.07^f respectively and retention time of 1.53 ± 0.01^b mins. and 3.59 ± 0.02^f mins. respectively (Table 3) (Figure 5).

Table 3. Peak area and retention time of Salidroside and Rosavin standard.

S.No.	Sample name	Weight (mg)	Peak area	Retention time (t_r) (mins.)
1.	Salidroside	-----	172320.5 ± 0.09^b	1.53 ± 0.01^b
2.	Rosavin	-----	1830682.5 ± 0.07^f	3.59 ± 0.02^f

<u>**NOTE**</u> **-** ANOVA test shows high significance with Duncan multiple-range test at 5% ($p=0.05$). Data shown are the means of triplicates $\pm$ standard error (SE).
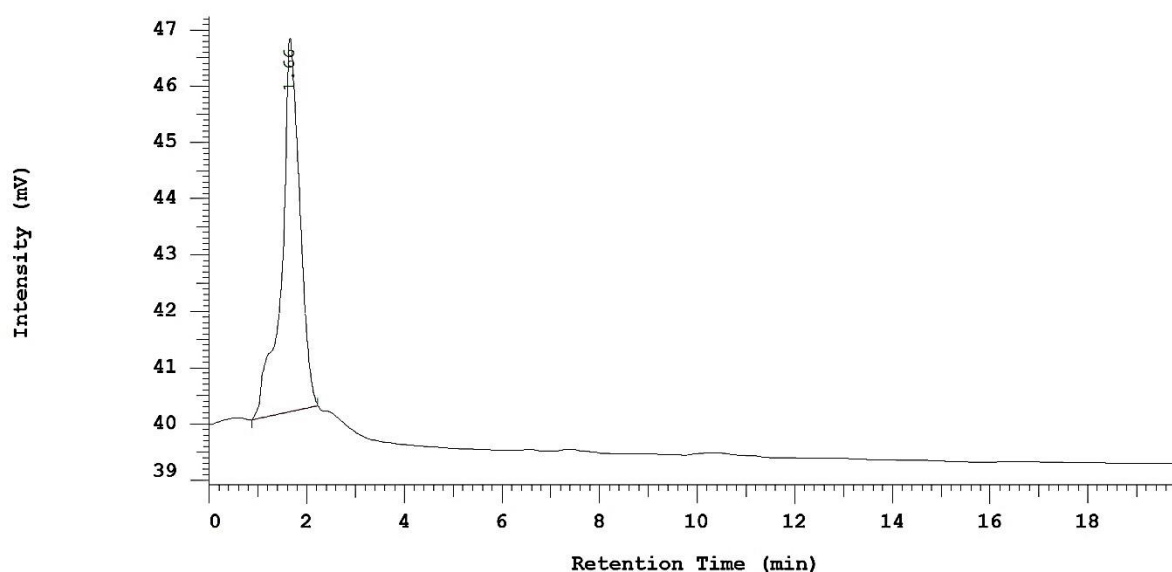
a.
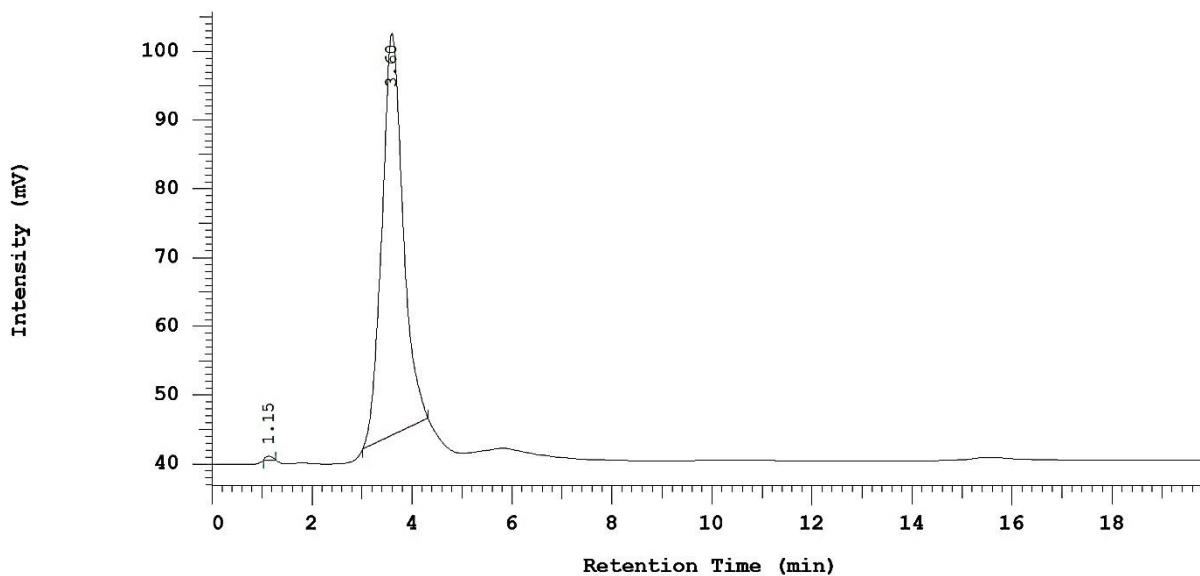
b.

Figure 5. Chromatograms for a.) Salidroside standard b.) Rosavin standard.

Quantification of Salidroside and Rosavin in callus and in vitro grown shoots and roots

The in vitro grown tissues were sampled periodically and were stored at -80 C for their quantification and estimation of Salidroside and Rosavin. Optimized HPLC conditions were used to quantify the samples. Salidroside was quantified in callus (green), in vitro grown shoots and in vitro grown roots as 2.401 ± 0.05^f µg/mg, 2.875 ± 0.08^d µg/mg and 0.712 ± 0.04^d µg/mg respectively (Figure 6) while no traces of Rosavin was found in these tissues (Table 4).

Table 4. Peak area, retention time and quantified amount of Salidroside and Rosavin in different tissues of *R. imbricata.*

S.No.	Sample name	Weight (mg)	Peak area	Retention time (t_r)	Amount quantified of Salidroside ($\mu g/mg$)	Amount quantified of Rosavin ($\mu g/mg$)
1.	Callus (green)	106.35	88017 ± 0.03^f	1.63 ± 0.01^f	2.401 ± 0.05^f	-----
2.	in vitro Shoots	103.60	102697.5 ± 0.17^d	1.66 ± 0.03^d	2.875 ± 0.08^d	-----
3.	In vitro Roots	101.30	24871 ± 0.11^d	1.55 ± 0.02^d	0.712 ± 0.04^d	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

a.

28
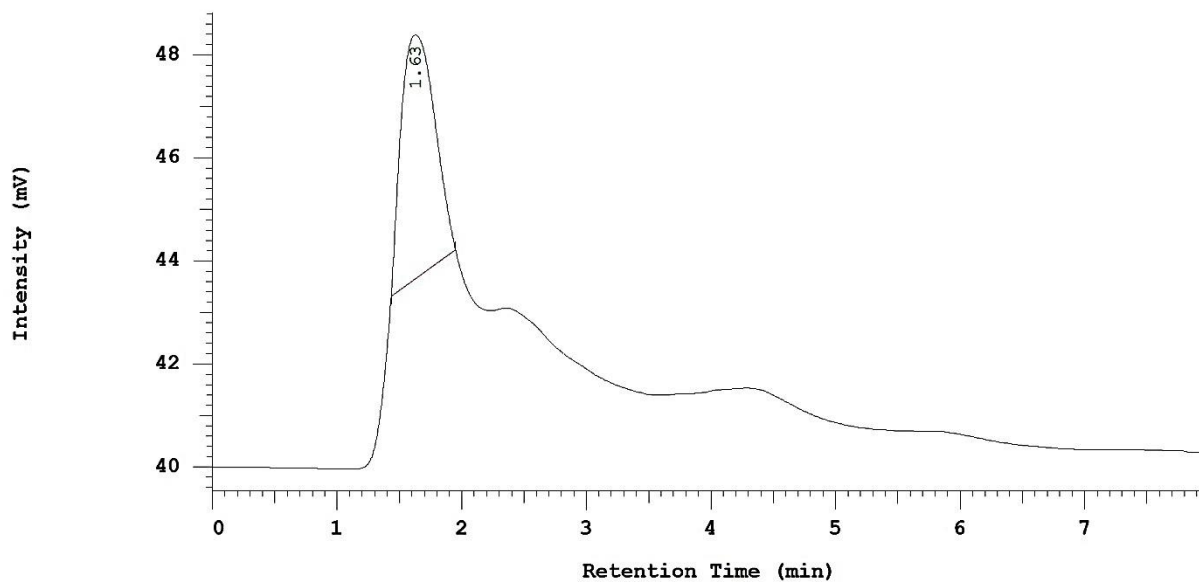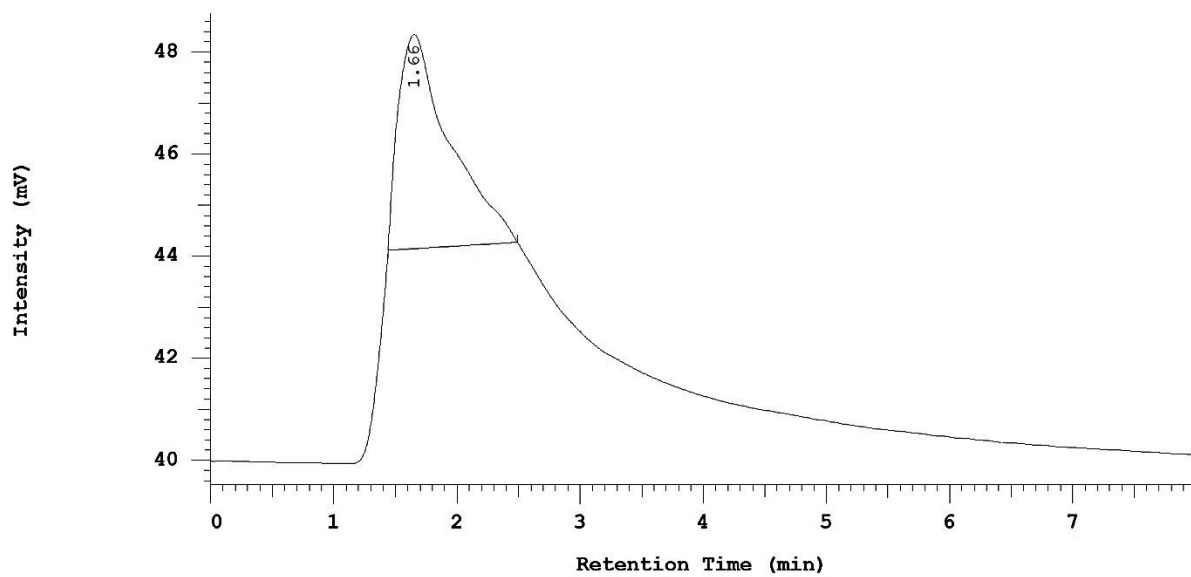
b.
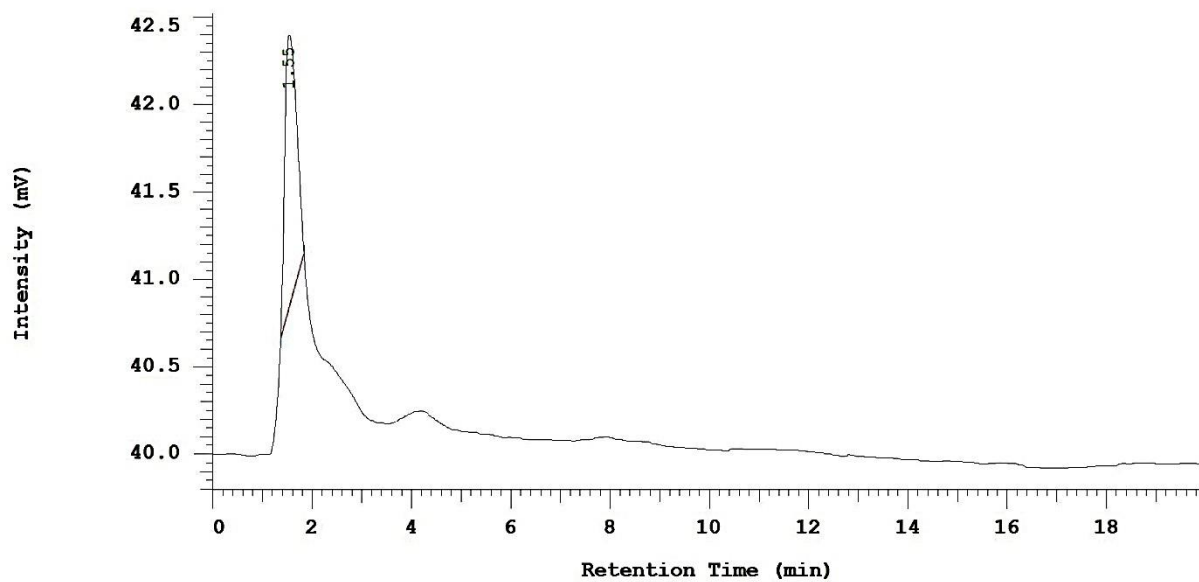
c.

Figure 6. Chromatograms for a.) Callus b.) in vitro Shoots c.) in vitro Roots of *R. imbricata* for quantification of Salidroside.

Development of contrasting conditions for accumulation of secondary metabolites and for transcriptome generation.

Optimization of MS media and growth hormones for accumulation of secondary metabolites in callus

Out of tested 10 media combinations, callus was induced in 6 media combination *viz.* RI2, RI3, RI4, RI5, RI6 and RI7 at $15 \pm 2°C$. MS medium having BAP (1 mg/l) + IBA (2 mg/l) was found to be superlative with callus induction in 10-35 days and 71-92% of calli percentage from leaf, intermodal segments and root explants at $15 \pm 2°C$ with 2.401 ± 0.05^f µg/mg of Salidroside whereas MS medium having BAP (1 mg/l) + IBA (2 mg/l) + GA$_3$ (2 mg/l) was found to be less efficient in callus induction with 25-40 days and 9-62 % of calli percentage from leaf, intermodal segments and root explants with 0.211 ± 0.04^b µg/mg of Salidroside (Table 5). Leaf explant was found to be unsurpassed for callus induction in 10-15 days with 92 ± 0.57^f % of calli percentage (Figure 7a, 7b, 7c). No trace of Rosavin was found in the samples.

a.   b.   c.

Figure 7. Callus induction from leaf explant of *R. imbricata.* a.) Leaf explant b.) Callus initiation (10-15 days) c.) Sub-cultured callus (25-30 days).

Table 5. Effect of MS media and various growth hormones on callus induction from leaf explant of _R.imbricata_ at 15±2°C along with the quantified amount of Salidroside.

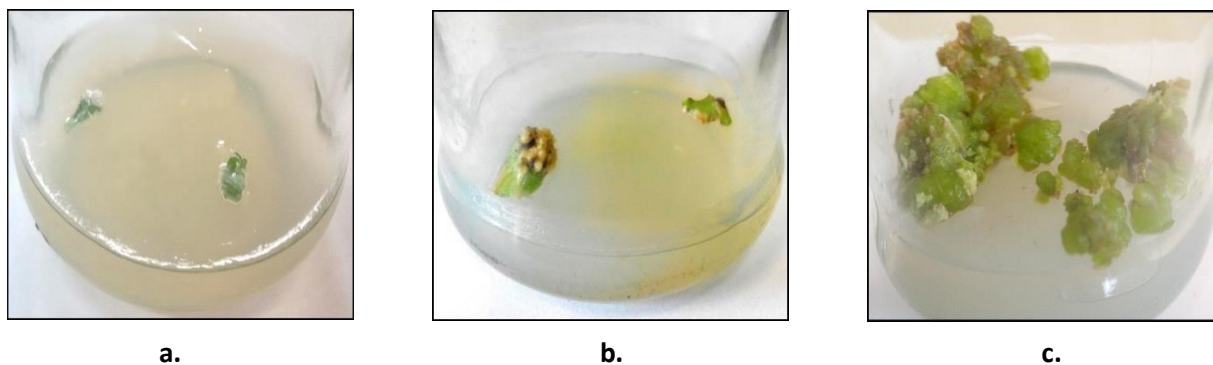| S.No.	Medium name	Days for callus induction	Callus colour	Percent calli (%)	Amount quantified (µg/mg)
1.	RI0	-----	-----	-----	-----
2.	RI1	-----	-----	-----	-----
3.	RI2	15-20	Cream	79±1.15[e]	1.874±0.11[e]
4.	**RI3**	**10-15**	**Green**	**92±0.57[f]**	**2.401±0.05[f]**
5.	RI4	15-20	Green	60±1.73[b]	1.062±0.03[b]
6.	RI5	25-30	Cream	62±1.15[b]	0.211±0.04[b]
7.	RI6	25-30	Green	74±1.15[d]	0.635±1.32[d]
8.	RI7	12-17	Green	68±1.73[c]	1.543±1.25[c]
9.	RI8	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

Optimization of MS media and growth hormones for accumulation of secondary metabolites in shoots

Out of tested 10 media combinations, shoot multiplication was commenced in 3 media combination _viz._ RI1, RI3, and RI7 at 15 ± 2°C. MS medium having BAP (1 mg/l) + KN (2 mg/l) was found to be the best for the shoot multiplication in 18-23 days with 4.74 ± 0.01[d] shoot number, average shoot length of 3.22 ± 0.01[d] at 15 ± 2°C (Figure 8a, 8b, 8c) along with 2.875 ± 0.08[d] µg/mg of Salidroside whereas MS medium having IBA (1 mg/l) + KN (3 mg/l) was found to be less efficient for the shoot multiplication in 20-25 days with 1.77 ± 0.00[b] shoot number, average shoot length of 2.17 ± 0.00[b] at 15 ± 2°C along with 0.314 ± 0.07[b] µg/mg of Salidroside (Table 6). No trace of Rosavin was found in the samples.

a.   b.   c.
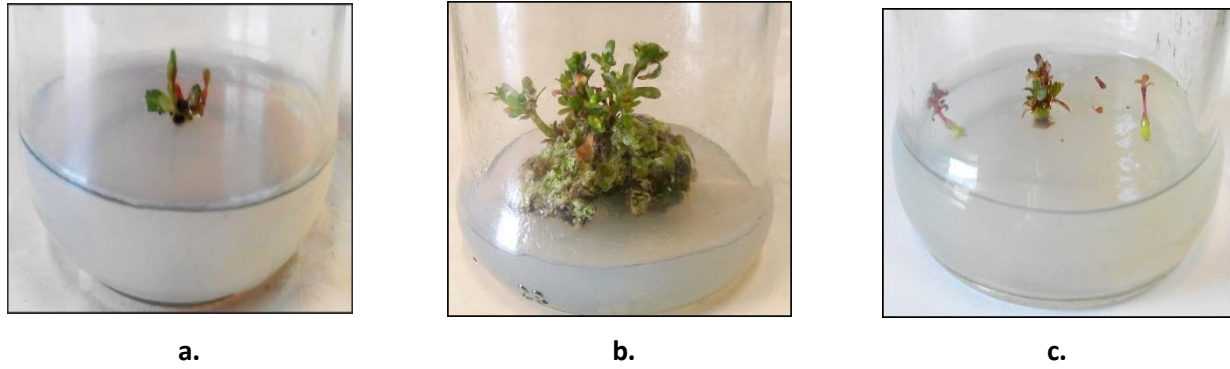
Figure 8. Direct shoot organogenesis from shoot apex of *R. imbricata.* a.) Shoot apex b.) Shoots formed after 20-25 days. c.) Shoot multiplication within 35 days.

Table 6. Effect of MS media and various growth hormones on shoot multiplication of *R. imbricata* at $15 \pm 2°C$ along with the quantified amount of Salidroside.

S.No.	Medium name	Days for shoot multiplication	Average no. of shoots	Average shoot length (cm)	Amount quantified (µg/mg)
1.	RI0	-----	-----	-----	-----
2.	**RI1**	**18-23**	**4.74±0.01[d]**	**3.22±0.01[d]**	**2.875±0.08[d]**
3.	RI2	-----	-----	-----	-----
4.	RI3	16-20	2.52±0.01[c]	2.72±0.00[c]	1.331±0.11[c]
5.	RI4	-----	-----	-----	-----
6.	RI5	-----	-----	-----	-----
7.	RI6	-----	-----	-----	-----
8.	RI7	20-25	1.77±0.00[b]	2.17±0.00[b]	0.314±0.07[b]
9.	RI8	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

Optimization of MS media and growth hormones for accumulation of secondary metabolites in roots

Out of tested 10 media combinations, rooting was obtained in 3 media combination *viz.*RI3, RI6 and RI7 at $15 \pm 2°C$. The MS medium having BAP (2 mg/l) + IBA (4 mg/l) was found to be the superlative for rooting in 25-30 days with 4.40 ± 0.11^d root number, average root length of 5.60 ± 0.17^d; 71.00 ± 0.57^d % of shoot forming roots at $15 \pm 2°C$ (Figure 9) along with 0.712 ± 0.04^d μg/mg of Salidroside whereas MS medium having with IBA (1 mg/l) + KN (3 mg/l) was found to be the less efficient for rooting in 40-45 days with 3.90 ± 0.11^b root number, average root length of 4.70 ± 0.11^b; 42.00 ± 2.30^b % of shoot forming roots at $15 \pm 2°C$ along with 0.122 ± 0.02^b μg/mg of Salidroside (Table 7). No trace of Rosavin was found in the samples.

Figure 9. Root formation (within 25-45 days) from the in vitro shoots of *R. imbricata.*

Table 7. Effect of MS media and various growth hormones on rooting of shoots of *R. imbricata* at $15 \pm 2°C$ along with the quantified amount of Salidroside.

S.No.	Medium name	Days for root initiation	Average no. of roots/shoot	Average root length (cm)	Percent shoot forming roots (%)	Amount quantified (µg/mg)
1.	RI0	-----	-----	-----	-----	-----
2.	RI1	-----	-----	-----	-----	-----
3.	RI2	-----	-----	-----	-----	-----
4.	RI3	35-40	4.10 ± 0.05^c	5.20 ± 0.11^c	63.00 ± 1.15^c	0.571 ± 0.35^c
5.	RI4	-----	-----	-----	-----	-----
6.	RI5	-----	-----	-----	-----	-----
7.	**RI6**	**25-30**	$\mathbf{4.40\pm0.11^d}$	$\mathbf{5.60\pm0.17^d}$	$\mathbf{71.00\pm0.57^d}$	$\mathbf{0.712\pm0.04^d}$
8.	RI7	40-45	3.90 ± 0.11^b	4.70 ± 0.11^b	42.00 ± 2.30^b	0.122 ± 0.02^b
9.	RI8	-----	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

Optimization of different temperature conditions for accumulation of secondary metabolites in callus

Callus growth, shoot multiplication and rooting were observed earlier at $15 \pm 2°C$ as compared to other temperatures including $10 \pm 2°C$, $25 \pm 2°C$ and $30 \pm 2°C$ along with higher Salidroside accumulation. MS medium having BAP (1 mg/l) + IBA (2 mg/l) was found to be unsurpassed for callus growth along with 2.401 ± 0.05^f µg/mg of Salidroside in callus at $15 \pm 2°C$ (Table 8), whereas, MS medium having BAP (1 mg/l) + KN (2 mg/l) was found to be the superlative for shoot multiplication along with 2.875 ± 0.08^d µg/mg of Salidroside in shoots at $15 \pm 2°C$ (Table 9). MS medium having BAP (2 mg/l) + IBA (4 mg/l) was found to be the preeminent for rooting along with 0.712 ± 0.04^d µg/mg of Salidroside in roots at $15 \pm 2°C$ (Table 10). No traces of Rosavin was found in any tissue at any temperature.

Table 8. Effect of MS media and various growth hormones on amount of Salidroside accumulation w.r.t. different temperatures in callus of *R. imbricata*.

S.No.	Medium name	Amount quantified at different temperatures (μg/mg)			
		10± 2°C	15 ± 2°C	25± 2°C	30± 2°C
1.	RI0	------	-----	------	------
2.	RI1	-----	-----	-----	-----
3.	RI2	1.522 ± 0.21^e	1.871 ± 0.11^e	1.313 ± 0.01^e	0.725 ± 0.03^e
4.	RI3	2.114 ± 0.02^f	$\mathbf{2.401\pm0.05^f}$	1.831 ± 0.11^f	0.927 ± 0.07^f
5.	RI4	0.773 ± 0.42^b	1.062 ± 0.03^b	0.522 ± 0.06^b	0.319 ± 0.04^b
6.	RI5	0.177 ± 0.02^b	0.211 ± 0.04^b	0.061 ± 0.15^b	0.008 ± 0.17^b
7.	RI6	0.416 ± 0.11^d	0.634 ± 0.02^d	0.268 ± 1.07^d	0.072 ± 0.12^d
8.	RI7	1.225 ± 0.72^c	1.544 ± 1.25^c	0.858 ± 0.57^c	0.542 ± 1.63^c
9.	RI8	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

Table 9. Effect of MS media and various growth hormones on amount of Salidroside accumulation w.r.t. different temperatures in shoots of *R. imbricata*.

S.No.	Medium name	Amount quantified at different temperatures (μg/mg)			
		10± 2°C	15 ± 2°C	25± 2°C	25± 2°C
1.	RI0	------	-----	------	------
2.	RI1	2.381 ± 0.02^d	$\mathbf{2.875\pm0.08^d}$	2.124 ± 0.01^d	1.782 ± 0.07^d
3.	RI2	-----	-----	-----	-----
4.	RI3	1.117 ± 0.09^c	1.334 ± 0.11^c	0.921 ± 0.03^c	0.573 ± 0.14^c
5.	RI4	-----	-----	-----	-----
6.	RI5	-----	-----	-----	-----
7.	RI6	-----	-----	-----	-----
8.	RI7	0.125 ± 0.04^b	0.314 ± 0.07^b	0.032 ± 0.12^b	0.005 ± 0.08^b
9.	RI8	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

Table 10. Effect of MS media and various growth hormones on amount of Salidroside accumulation w.r.t. different temperatures in roots of *R. imbricata*.

S.No.	Medium name	Amount quantified at different temperatures (µg/mg)			
		10± 2°C	15 ± 2°C	25± 2°C	25± 2°C
1.	RI0	------	-----	------	------
2.	RI1	-----	-----	-----	-----
3.	RI2	-----	-----	-----	-----
4.	RI3	$0.331{\pm}0.03^c$	$0.571{\pm}0.35^c$	$0.275{\pm}0.07^c$	$0.103{\pm}0.04^c$
5.	RI4	-----	-----	-----	-----
6.	RI5	-----	-----	-----	-----
7.	RI6	$0.431{\pm}0.01^d$	$\mathbf{0.712{\pm}0.04^d}$	$0.212{\pm}0.09^d$	$0.119{\pm}0.02^d$
8.	RI7	$0.102{\pm}0.14^b$	$0.122{\pm}0.02^b$	$0.087{\pm}0.11^b$	$0.052{\pm}0.08^b$
9.	RI8	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----

NOTE - ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data shown are the means of triplicates ± standard error (SE).

Identification of key genes for Salidroside and Rosavin biosynthesis

RNA Isolation from *R. imbricata*

RNA isolated from in vitro grown Shoots and Callus (green) of *R. imbricata* were run on 1% agarose gel and two intact bands of 28s and 18s were observed (Figure 10). The isolated RNA were also quantified using Nano drop accessory of Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific) and the data obtained is depicted in Table 11.

Figure 10. RNA isolated from in vitro grown Shoots and Callus of *R. imbricata*.

Table 11. Quantification data of RNA isolated from Callus and Shoots of *R. imbricata*.

S.No.	Explant	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
1.	Callus	253	1.93	1.72
2.	in vitro Shoots	281	1.91	1.77

cDNA Synthesis and check

Synthesized cDNA was checked by PCR amplification using 26s rRNA primers. Amplified products were run on 1% agarose gel and an intact bright bands 26s rRNA were observed at 500bp (Figure 11). The synthesized cDNA were also quantified using Nano drop accessory of Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific) and the data obtained is depicted in Table 12.
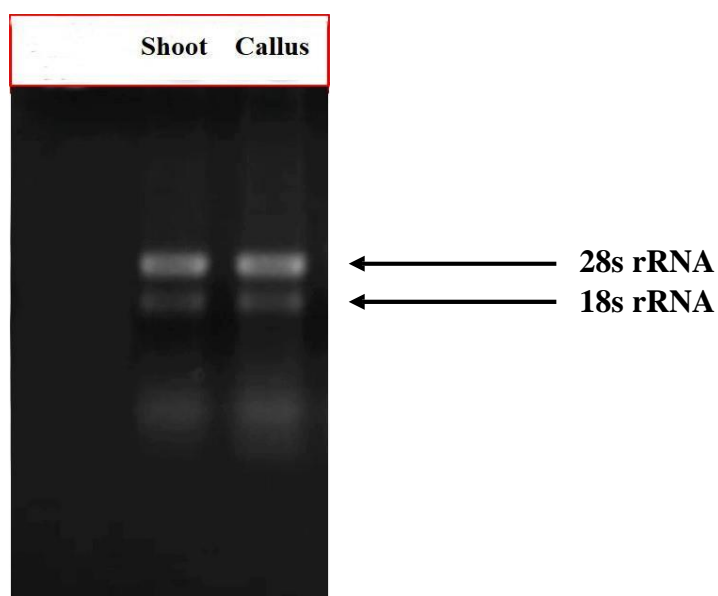

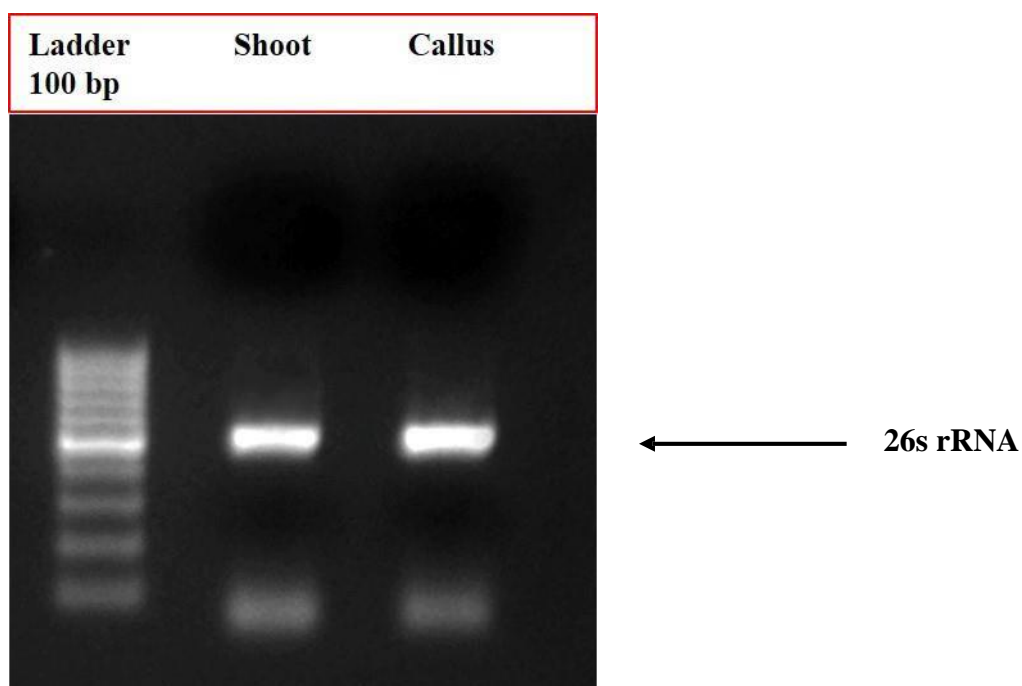**Figure 11.** cDNA from in vitro grown Shoots and Callus of *R. imbricata* amplified using 26s rRNA primers.

Table 12. Quantification data of cDNA synthesized from RNA isolated from Callus and Shoots of *R. imbricata.*

S.No.	RNA Source	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
1.	Callus	668	1.92	1.74
2.	in vitro Shoots	704	1.95	1.71

Primers Designing for Salidroside biosynthesis genes

Primers sequences designed for key genes responsible for biosynthesis of Salidroside are listed in Table 13. along with the T_m and amplicon size.

Table 13. Primers Designed for Genes responsible for biosynthesis of Salidroside in *R. imbricata*.

Genes	Designed Primer Sequence	Amplicon Size
TyrDC	F- TBCCDGGSMTMACHCATTGGCAAA	536 bp
	R- GTYGTBCCAAYHGTBSCRCAHANG	
UDPGT	F- TVAATWSBTTYBWVGASYTDGA	501 bp
	R- TGDSMARCWATRTAAGGYTCRGT	
β-GD	F- GAYTTYTATMAYCGWTWCRARGADGA	464 bp
	R- TGDSMARCWATRTAAGGYTCRGT	
AAD	F- TTYGACAYSKCSGATYHCTATGGGC	313 bp
	R- ASCWAYRKKCTTGATTTTMCCTTCT	

Primers Designing for Rosavin biosynthesis genes

Primers sequences designed for key genes responsible for biosynthesis of Rosavin are listed in Table 14. along with the T_m, and amplicon size.

Table 14. Primers Designed for Genes responsible for biosynthesis of Rosavin in *R. imbricata*.

Genes	Designed Primer Sequence	Amplicon Size
PAL	F- ACDTCTCCHCAATGGBTDGGBCCT	413 bp
	R- GAGTTSACATCYTGGTTRTGYTGC	
4CL	F- ACNACVGGRYTRCCAAARGGRGTS	436 bp
	R- CVGTCATHCCRTADCCCTGWCCVA	

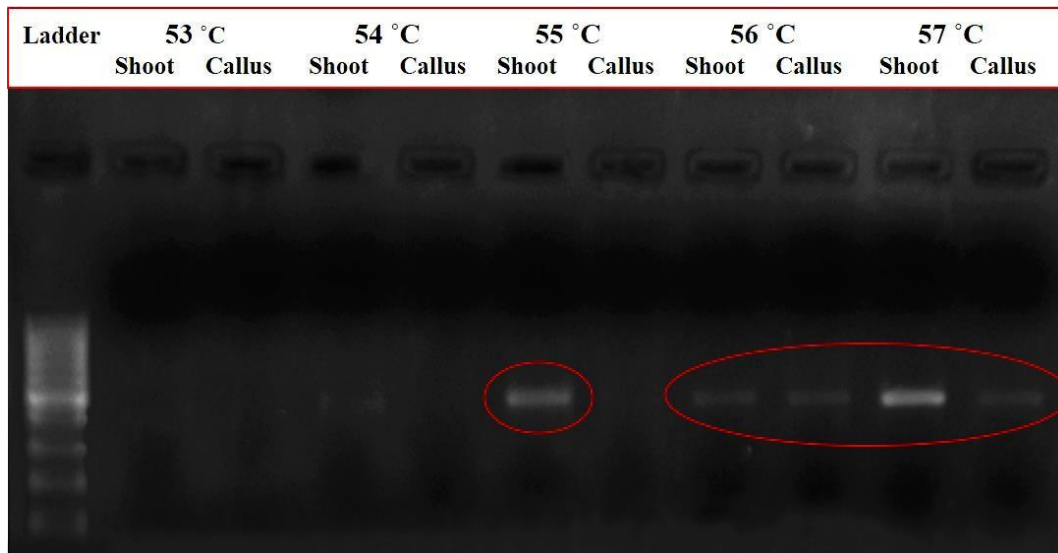|			
CCR	F- TAYCCDWTGGTYCCHGGVCATGA		399 bp
	R- ATGTGNCCWASWCCWCCDAGYCC		
CAD	F- TTCTGCAARAAYACYAAGAATTGG		372 bp
	R- CTTGGTRGGDABDGGRTACTCSGG		

PCR Amplification

After completion of PCR cycles, samples were run on 1.2% agarose gel (Figure 12) and T_m optimized for each primer is listed in Table 15.

Table 15. Optimized T_m and Amplicon size of the amplified Genes responsible for biosynthesis of Salidroside and Rosavin in *R. imbricata*.

Metabolite	Genes	T_m (C)	Amplicon Size
	TyrDC	50.5	536 bp
	UDPGT	57.0	501 bp
SALIDROSIDE	β-GD	53.0	464 bp
	AAD	54.0	313 bp
	PAL	59.0	413 bp
	4CL	59.0	436 bp
ROSAVIN	CCR	58.0	399 bp
	CAD	58.0	372 bp

a.

b.

c.

d.

e.

Shoot

Callus

| 57 °C | 59 °C | 60 °C | 61 °C | 63 °C | | 57 °C | 59 °C | 60°C | 61 °C | 63 °C | Ladder |

f.

Shoot

Callus

| Ladder | 55 °C | 56 °C | 57 °C | 58 °C | 59 °C | | 55 °C | 56 °C | 57 °C | 58 °C | 59 °C |

g.

43
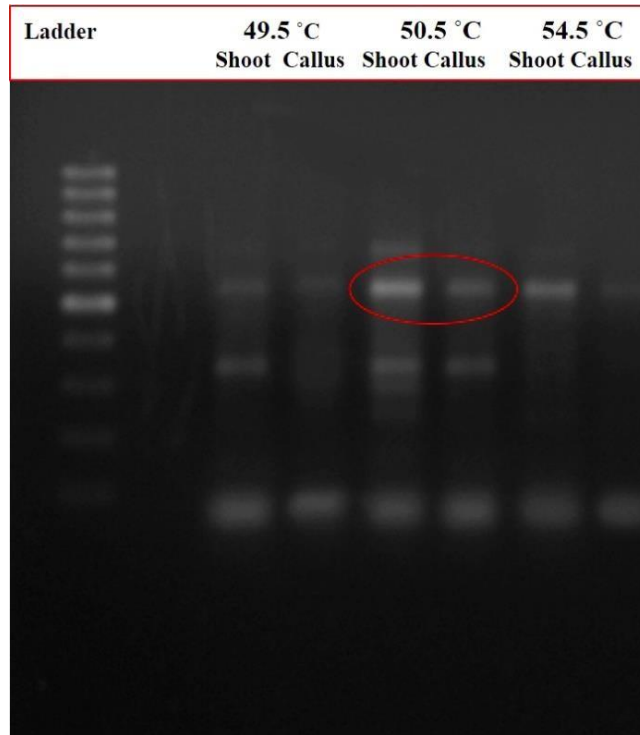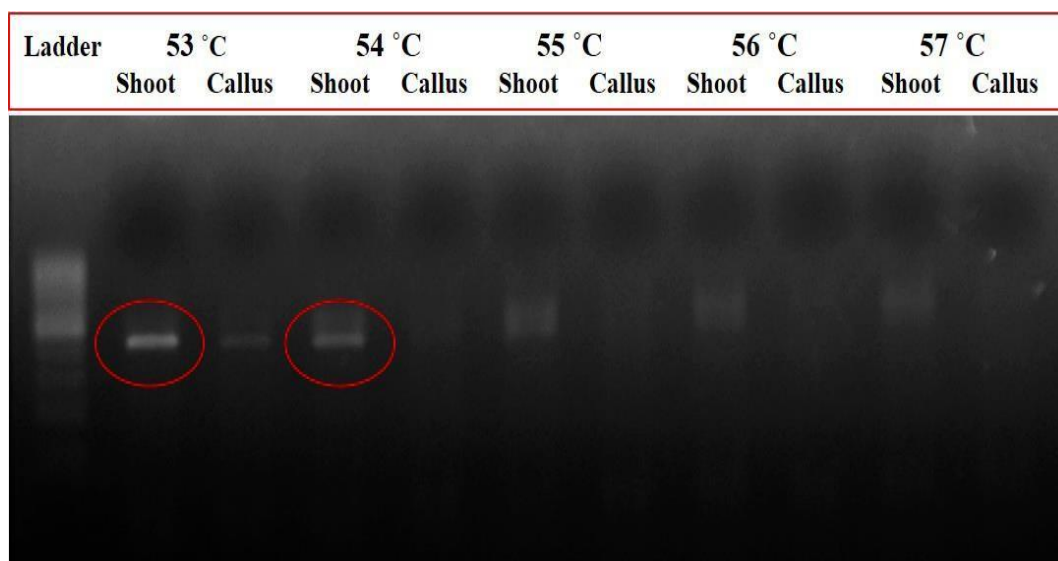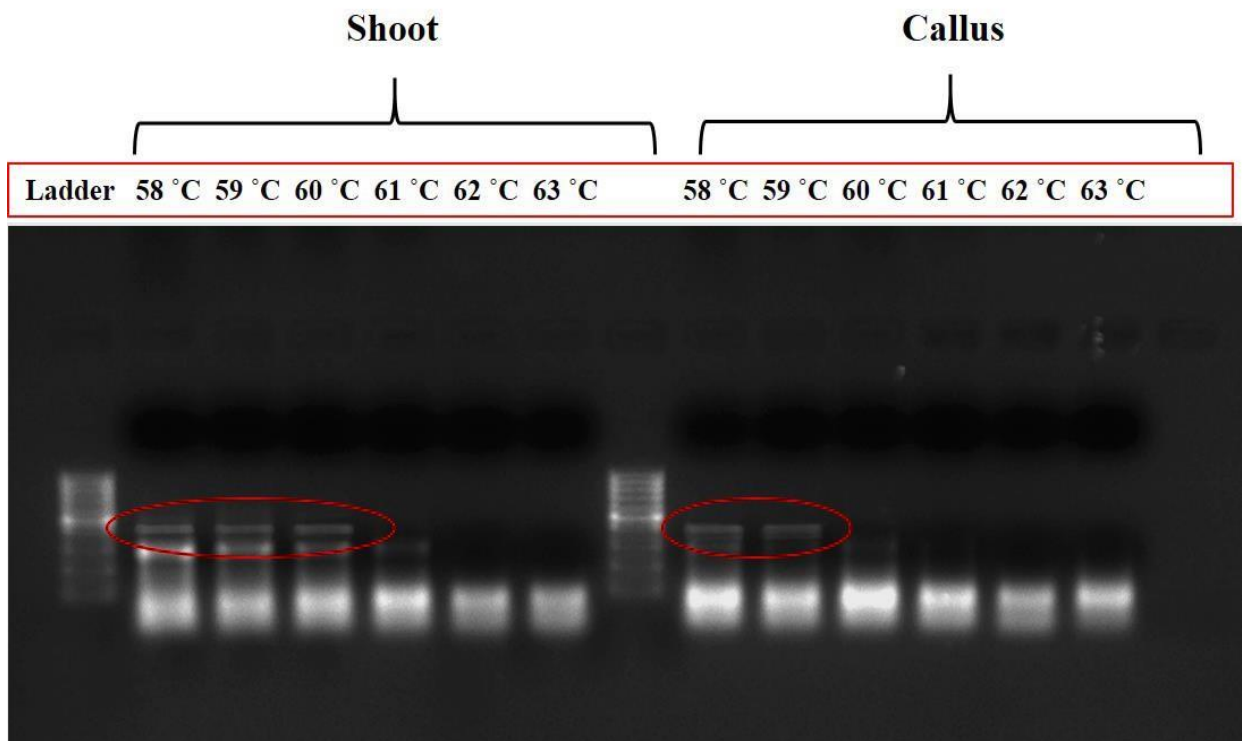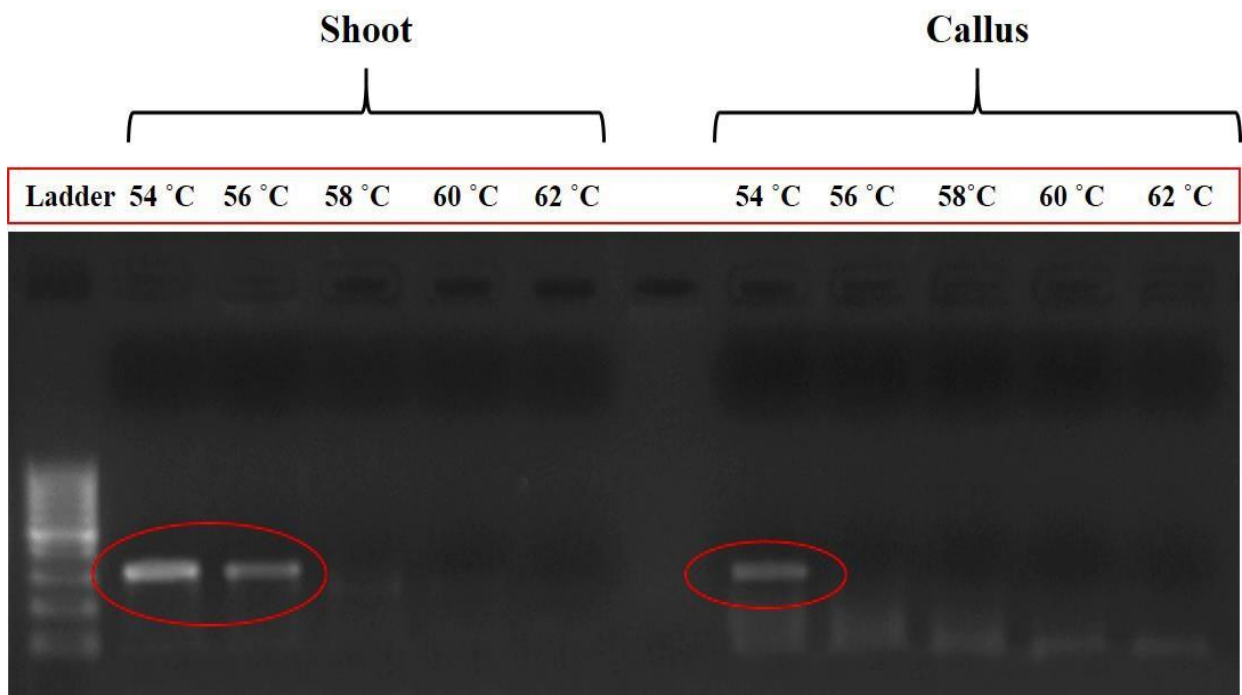
h.

Figure 12. Gel pics of amplified genes responsible for biosynthesis of Salidroside and Rosavin at different T$_m$ in *R. imbricata*. a.) TyrDC b.) UDPGT c.) β-GD d.) AAD e.) PAL f.) 4CL g.) CCR h.) CAD

DISCUSSION

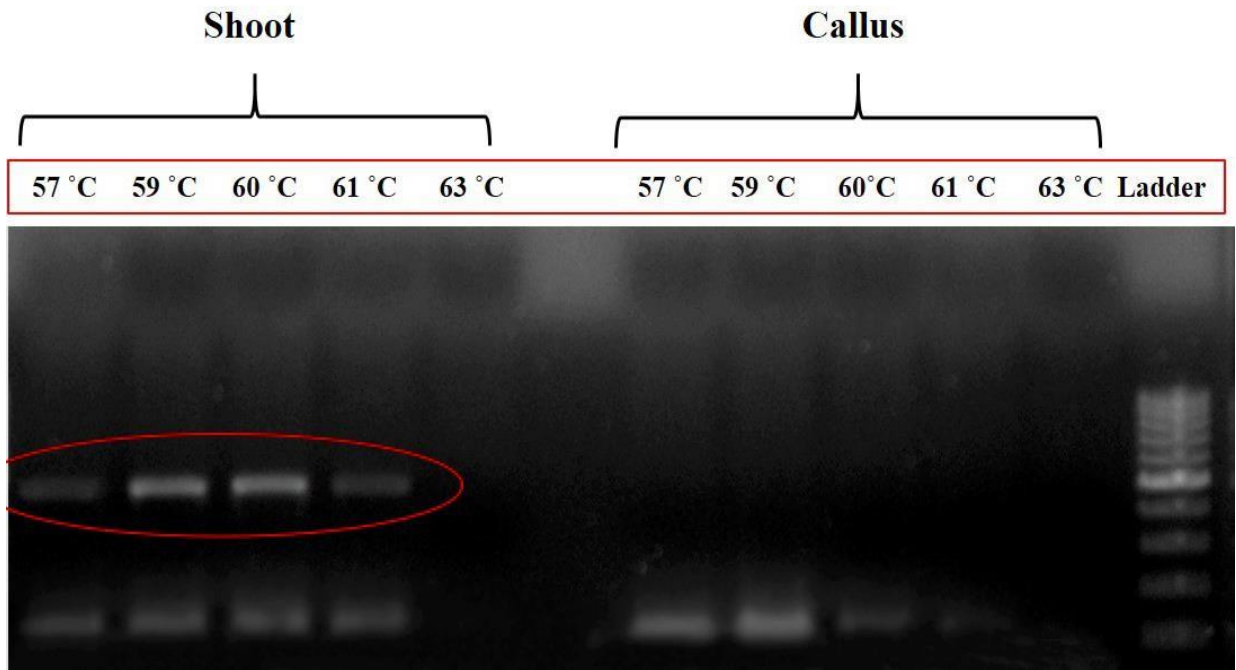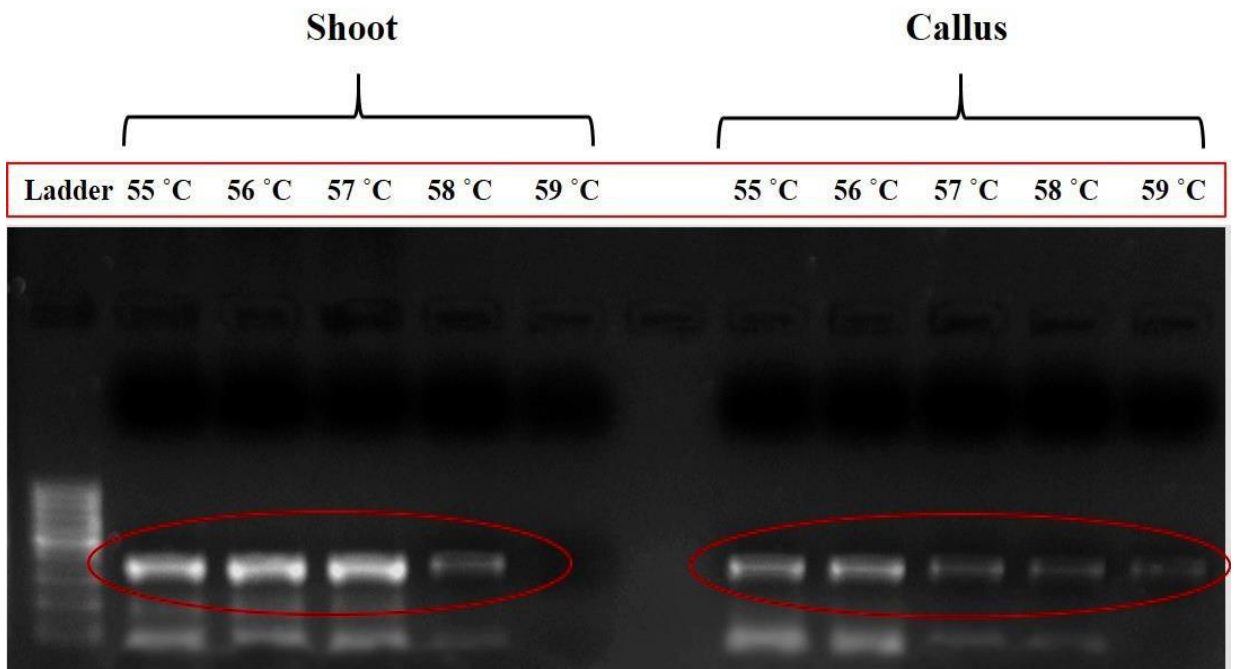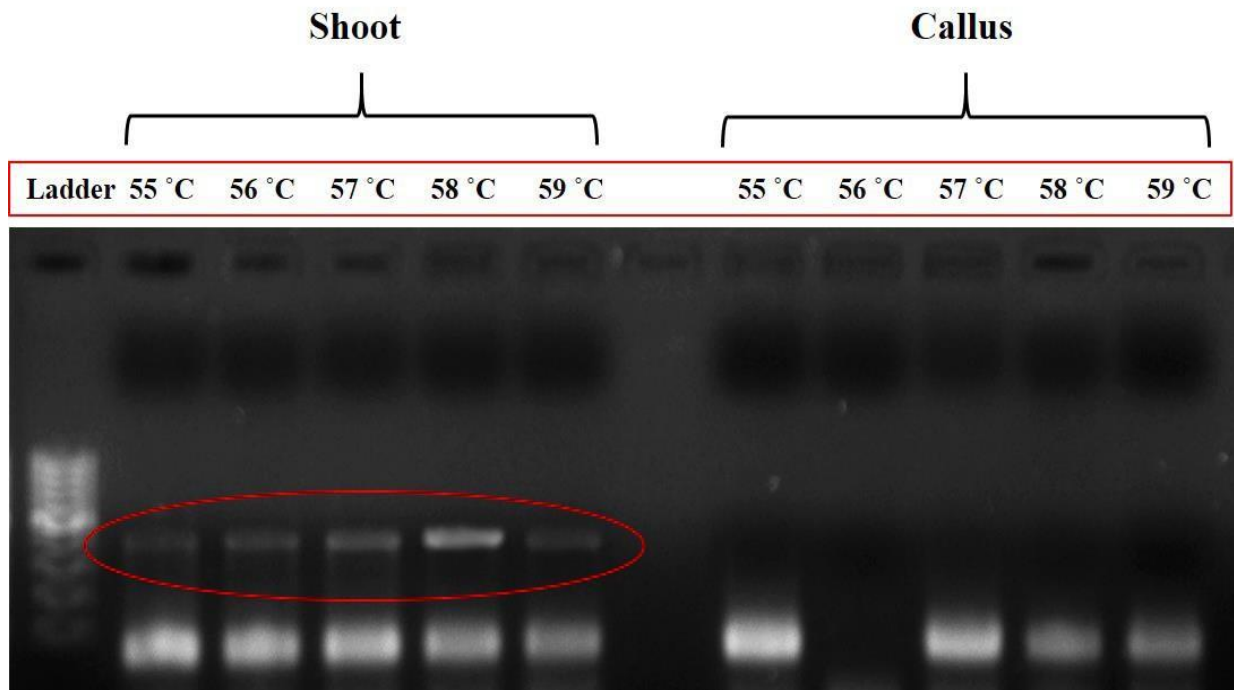The objectives of this study were to optimize the HPLC conditions and quantification of two pharmacologically important compounds viz. Salidroside and Rosavin in callus, in vitro shoots and in vitro roots from cultures maintained at $15 \pm 2°C$; identification of genes responsible for biosynthesis of Salidroside and Rosavin; and development of contrasting conditions by optimizing MS media & growth hormones and different temperatures for accumulation of secondary metabolites for transcriptome generation.

People have reported that in medicinal and aromatic plants, the medicinally important metabolites are biosynthesized and accumulate in different organs such as roots, leaves and shoots (Ramachandra and Ravishankar, 2002). Similarly, accumulation of medicinal compounds do occur in rhizome of *R. imbricata* (Mishra et al., 2008).

The HPLC conditions for quantification of in vitro grown callus shoots and roots for the presence of Salidroside and Rosavin were optimized. It was found that the optimum resolution could be achieved using 0.16 M ammonium acetate buffer (B), adjusted to pH 5.6 and 100% methanol (A) as mobile phase with isocratic elution. Similarly, Ma et al., 2008 used methanol and ammonium acetate buffer for quantification of Salidroside, p-tyrosol, rosarian, rosavin, rosin, and rosiridin in *Rhodiola spp*. Upon quantification of secondary metabolites in in vitro grown callus, shoots and roots maintained at $15 \pm 2°C$, Salidroside was found to be as 2.401 ± 0.05^f µg/mg, 2.875 ± 0.08^d µg/mg and 0.712 ± 0.04^d µg/mg of Fresh weight respectively while no traces of Rosavin was found in these tissues (Figure 6) (Table 4). People have quantified Salidroside in callus of various *Rhodiola spp.* ranging from 0.16 µg/mg to 26.48 µg/mg of DW (Grech-Buran et al., 2015), whereas, in this study, fresh weight of samples were used to quantified the Salidroside. Furmanov et al., (1998) found that Salidroside was present in organs of intact *Rhodiola rosea* plant while Rosavin was present in roots of different origin of *R. rosea* as well as shoots of intact *R. rosea* plants. They also proposed that the Rosin (precursor for Rosavin) might get transformed into p- hydroxyrosin (= triandrin) in in vitro cultures of *R. rosea* by the action of phenylhydroxylases. That is why, triandrin was more distinctive for in vitro tissue cultures. In 14 different species of *Rhodiola* L., significant differences were found for the Salidroside contents in field grown rhizome. Salidroside was present in all the selected plants but their levels ranged between 0.02 µg/mg (*R. sinuate*) and 15.95 µg/mg (*R. sacra*) (Chen et al., 2008).

Contrasting conditions using MS media & different growth hormones combinations for the accumulation of secondary metabolites and for transcriptome generation were also developed

in this study. It was found that the MS medium having BAP (1 mg/l) + IBA (2 mg/l) was superlative with callus induction in 10-35 days and 71-92% of calli percentage from leaf (Figure 7a, 7b, 7c), intermodal segments and root explants at $15 \pm 2°C$ with 2.401 ± 0.05^f µg/mg of Salidroside (Table 5). Sharif et al., (2016) also showed that MS media having IBA and BAP lead to highest callus weight from broccoli leaf slice. Different explants respond differently for regeneration depending upon the endogenous concentration of growth hormones under similar set of in vitro conditions (Sharma et al., 2016). We have also earlier reported that leaf was found to be the unsurpassed explant for callus induction (Sharma et al., 2016). Likewise, it was found that the MS medium having BAP (1 mg/l) + KN (2 mg/l) was the superlative for shoot multiplication in 18-23 days with 4.74 ± 0.01^d shoot number, average shoot length of 3.22 ± 0.01^d at $15\pm2°C$ (Figure 8a, 8b, 8c) along with 2.875 ± 0.08^d µg/mg of Salidroside (Table 6). Sharma et al., (2016) have shown better growth with high secondary metabolite content in *Picrorhiza kurroa* plants at $15 \pm 2°C$ compared to $25 \pm 2°$ C. We have also reported that MS medium having BAP (1 mg/l) + KN (2 mg/l) was found to be superlative for the shoot multiplication (Sharma et al., 2016). It was also found that the MS medium having BAP (2 mg/l) + IBA (4 mg/l) was found to be the preeminent for rooting in 25-30 days with 4.40 ± 0.11^d root number, average root length of 5.60 ± 0.17^d; 71.00 ± 0.57^d % of shoot forming roots at $15 \pm 2°C$ (Figure 9) along with 0.712 ± 0.04^d µg/mg of Salidroside (Table 7). Sharif et al., (2015) also showed that MS media having IBA and BAP produce root proliferation from broccoli using root tip cultures. We have also reported that MS medium having BAP (2 mg/l) + IBA (4 mg/l) be superlative for the root formation (Sharma et al., 2016). All of these above mentioned contrasting conditions were maintained at $15 \pm 2°C$.

Contrasting conditions using different temperatures for the accumulation of secondary metabolites and for transcriptome generation were also developed. It was found that callus growth, shoot multiplication and rooting were observed earlier at $15 \pm 2°C$ as compared to other temperatures including $10 \pm 2°C$, $25 \pm 2°C$ and $30 \pm 2°C$ along with higher Salidroside accumulation i.e 2.401 ± 0.05^f µg/mg, 2.875 ± 0.08^d µg/mg and 0.712 ± 0.04^d µg/mg respectively (Table 8, 9, 10). Similarly, maximum anthocyanin content obtained at $15°C$ was about 13-fold higher than the content obtained at $35°C$ for strawberry cell culture (Zhang et al., 1997). Also, the native growth conditions at low temperatures for the field propagation of *Rhodiola imbricata* can be accounted for higher Salidroside amount in callus, shoot and roots from cultures maintained at $15 \pm 2°C$. No traces of Rosavin was found in any tissue at any temperature. This maybe be due to negligible or trace amount of Rosavin accumulation under in vitro conditions as opposed to high accumulation in field conditions or due to break down of Rosavin in in vitro culture of *Rhodiola spp.* as quoted by Kurkin et al., (1991).

Also, designing of primers (Table 13, 14), optimizing the T_m for the designed primers (Table 15) and amplifying the genes (Figure 12) responsible for biosynthesis of Salidroside and Rosavin in in vitro callus and shoots were accomplished. Lan at al., (2013) concludes that tyrosine decarboxylase (TyrDC) played a crucial role in biosynthesis of Salidroside as the gene expressing levels of TyrDC along with UDPGT were consistent with the levels of Salidroside accumulation in *R. crenulata*. Furthermore, biosynthesis of tyramine was promoted by TyrDC overexpression that facilitated more metabolic flux flowing toward the downstream pathway. As a result, the intermediate tyrosol was accumulated in higher amount that that led to the improved Salidroside production. Shi Ling-ling et al., 2007, proposed that β-D-Glucosidase may have a dual nature as it may act though anabolic reaction by adding glucose to the tyrosol to produce Salidroside or may act through catabolic reaction by removing glucose from Salidroside to form tyrosol.

The *R. imbricata* is an endangered medicinal herb which warrants its molecular exploration for the biosynthesis of metabolites under in vitro conditions as has been reported in plentiful medicinal plants in order to relieve burden from its natural habitat (Vanisree et al., 2004). Also due to the presence of various pharmacological activities, *R. imbricata* is having potential use pharmaceutical industries as raw materials to develop formulations for increasing stamina, work productivity, longevity and to decrease fatigue, stress and depression. These developed protocols can be used for further extensive molecular dissection for secondary metabolites like Salidroside, Rosavin, which are of high demand.

CHAPTER - 5

CONCLUSION

AND FUTURE

WORK

R. imbricata is also known as golden root because of its ample pharmacological activities due to the presence of secondary metabolites in it. So, we have optimized the HPLC conditions for the quantification of Salidroside, which came out to be 2.401 ± 0.05^f µg/mg, 2.875 ± 0.08^d µg/mg and 0.712 ± 0.04^d µg/mg in in vitro grown callus, shoots and roots maintained at $15 \pm 2°C$. Though, Rosavin has not been quantified which may be due to its transformation to some other compound in in vitro conditions or due to its presence in very negligible amounts. Further experimentations can be done where we can find those developmental stages where the Rosavin can be also accumulated. We have also developed the contrasting condition using MS media & various growth hormones and different temperatures to find the media combination and temperature where the maximum amount of metabolite gets accumulated. It was found that in callus maintained at $15 \pm 2°C$, MS medium containing BAP (1 mg/l) + IBA (2 mg/l) resulted in 2.401 ± 0.05^f µg/mg of Salidroside, while in in vitro shoots maintained at $15 \pm 2°C$, MS medium containing BAP (1 mg/l) + KN (2 mg/l) resulted in 2.875 ± 0.08^d µg/mg of Salidroside. Also in in vitro roots of plantlets maintained at $15 \pm 2°C$, MS medium having BAP (2 mg/l) + IBA (4 mg/l) resulted in 0.712 ± 0.04^d µg/mg of Salidroside. It was also found that callus growth, shoot multiplication and rooting were observed earlier at $15 \pm 2°C$ as compared to other temperatures including $10 \pm 2°C$, $25 \pm 2°C$ and $30 \pm 2°C$ along with higher Salidroside accumulation. These conditions can be further used for transcriptome analysis and other molecular studies. We have also identified the genes responsible for biosynthesis of Salidroside and Rosavin in callus and in vitro shoots which could provide a lead for extensive molecular interventions. Once the sequences of amplified genes are obtained, these can be used for expression studies to analyze the expression of these genes under the mentioned contrasting conditions. Furthermore, overexpression of these genes can be done using elicitors or other molecular interventions in order to upsurge the amount of these secondary metabolites. This study can assist in the large scale production of Salidroside and Rosavin at commercial level in order to meet the demand of herbal and pharmaceutical industries.

LIST OF PUBLICATIONS

INTERNATIONAL AND NATIONAL JOURNAL PUBLICATIONS

- Anaida Kad, Archit Pundir, **Shubham Sharma**, Hemant Sood (2017). **Development of suspension cultures and ex-vitro rooting in Rauwolfia serpentina for rapid and large scale multiplication.** *International Journal of Innovative Research in Science and Engineering* 3(1): 135-143. ISSN 2454-9665 (IDIIF Impact Factor – 2.03)

- **Shubham Sharma**, Devanshi Popli, Neha Sharma, O. P Chaurasia, Hemant Sood (2016). **Effect of temperature on in vitro organogenesis of Rhodiola imbricata EDGEW. – A Medicinal Herb.** *World Journal of Pharmacy and Pharmaceutical Sciences* 5(12): 1228-1242. ISSN 2278-4357 (SJIF Impact Factor – 6.041) [DOI : 10.20959/wjpps201612-8266]

- Devanshi popli, **Shubham Sharma**, Hemant Sood (2016). **Optimization of liquid MS medium for enriching biomass of Dactylorhiza hatagirea.** *International Journal of Tropical Agriculture* 34(5): 1271-1276. ISSN 0254-8755 (NAAS – 3.03)

CONFERENCES/PAPER PRESENTATION/POSTER PRESENTATION

- Anaida Kad, Archit Pundir, **Shubham Sharma**, Hemant Sood (2017). **Development of suspension cultures and ex-vitro rooting in** *Rauwolfia serpentina* **for rapid and large scale multiplication.** *Proceedings of International Conference on Innovative Trends in Science, Engineering and Management* [**3rd**: New Delhi, India: 7th January] (Paper Presentation)

- **Shubham Sharma,** Sahil Kapoor, Neha Sharma, Rajinder S. Chauhan, O P Chaurasia, Hemant Sood (2016). **Callus induction and shoot regeneration for conservation of high value medicinal plant** *Rhodiola imbricata.* *Proceedings of the International Conference on Biotechnology and Bioinformatics* [**3rd**: Pune, India: 5-7 February], pp. - 212 – 215. (Poster Presentation & Abstract)

Certificate of Appreciation for Best project and thesis for the academic session **2015-2016** by **Dr. Shashi Kumar** (Group Leader, Metabolic Engineering), **International Centre for Genetic Engineering and Biotechnology (ICGEB)**, New Delhi, India and internal evaluation committee of Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, H.P., India.

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