OPTIMIZATION OF TISSUE CULTURE CONDITIONS FOR THE PROPAGATION OF *RHODIOLA IMBRICATA EDGEW.*

ENROLLMENT NO. **123820**

NAME OF STUDENT **SHUBHAM SHARMA**

NAME OF SUPERVISOR **DR. HEMANT SOOD**

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WAKNAGHAT

TABLE OF CONTENT

CERTIFICATE FROM SUPERVISOR

This is to certify that the work titled "**Optimization of tissue culture conditions for the propagation of *Rhodiola imbricata Edgew.***" submitted by "**Shubham Sharma**" in partial fulfillment for the award of degree of B.Tech of 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.

Signature of Supervisor -

Name of Supervisor - Dr. Hemant Sood

Designation - Assistant Professor (Senior Grade)

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat

Date -

DECLARATION

I certify that

- The work contained in this thesis is original and has been done 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.

Signature of the student -

Name of Student - Shubham Sharma

Enrollment No. - 123820

Date -

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Signature of the student -

Name of Student - Shubham Sharma

Enrollment No. - 123820

Date -

SUMMARY

Rhodiola imbricata Edgew. is a perennial herb of the family Crassulaceae, found in extreme Himalayan region at an altitude of 4000–5000 m. It is also available in border regions of Leh – Ladakh, China and Tibet. *R. imbricata* has been used extensively for its medicinal properties in traditional folk medicine in China, Tibet, Mongolia and the former Soviet Republics to increase physical endurance, work productivity, longevity and to treat fatigue, asthma, haemorrhage, impotence 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 flavonoids, coumarins and phenyl glycosides (p-tyrosol, salidroside, rosavin and rosin). To the best of our knowledge, this is the first report on micropropagation of *R. imbricata.* In this study, 10 different media combinations having different concentrations of IBA, KN, BAP, GA_3 and TDZ were tested for *in vitro* multiplication from different explants. Results revealed that MS media supplemented with BAP (1 mg/L) + IBA (2 mg/L) was found to be best for callus initiation, indirect shoot regeneration, while the best direct shoot organogenesis and multiplication was achieved in MS media having BAP (1 mg/L) + KN (2 mg/L) under optimized tissue culture conditions. MS media supplemented with BAP (2 mg/L) + IBA (4 mg/L) was found to be best for root initiation. Therefore, this study will provide a platform to meet high demand of raw material worldwide.

Signature of Student

Name - Shubham Sharma

Date -

Signature of Supervisor

Name - Dr. Hemant Sood

Date -

LIST OF FIGURES

LIST OF TABLES

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 consists of over 1400 species distributed 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 grows in drained soil at an altitude of 2700-5000 m and has been known for long to possess immense medicinal potential (Mishra et al., 2007). The *Rhodiola*, consisting of about 90 species, are widely distributed in the high altitude cold desert region of the Northern Hemisphere (Yidong Lei, et al., 2003). Many *Rhodiola* species have been used in traditional medicines for the treatment of long term illness and weakness due to infection in Tibet and other regions for over 1000 years (Xiong, 1995; Rohloff, 2002).

In India, there are six species of *Rhodiola*, namely *R. heterodonta, R. imbricata, R. quadrifida, R. sinuate, R. tibetica, and R. wallichiana* (Chaurasia and Gurmet, 2003). *Rhodiola imbricata Edgew*. previously known as Sedum roseum (Linn.) Scop., belonging to the plant family Crassulaceae. It is a succulent perennial herb 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 and rhizomes are the used parts of the plant. The root of R. imbricata has been getting a lot of attention lately. 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, herbaceous perennial plant, originating in the mountain regions of South West China and native around whole of the Northern hemisphere (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 mountain regions of Eurasia (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, golden outside, pink 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 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. The plant *R. imbricata* from trans-Himalayan cold desert of Ladakh region, India (Tayade 2015)

Analysis of different species of genus *Rhodiola*, chemical composition revealed six groups of active principles (Khanum et al., 2005). Phenylpropanoids: rosavin, rosin, rosarin. The name rosavin includes these three. Phenylethanol derivatives: rhodioloside (salidroside, rhodosin), tyrosol. Flavonoids: rhodiolin, rhodionin, rhodiosin, acetylrhodalgin, tricin. Monoterpenes:

rosiridol. Triterpenes: daucosterol, β-sitosterol. Phenol acids: chlorogenic, hydroxycinnamic 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).

Rhodiola species have been used as traditional medicines for the treatment of long term illness and weakness due to infection in Tibet and other regions for over 1000 years (Xiong, 1995; Rohloff, 2002). *R. imbricata* is an important and widely used food crop and traditional medicinal plant distributed in trans-Himalayan cold desert regions. In Amchi and Tibetian system of traditional medicine, the roots are used against lung problems, cold, cough, fever, loss of energy and pulmonary complaints (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) stress induced hypothermia and post stress recovery: mechanism and action (Gupta et al., 2009), anti- proliferative effects (Mishra et al., 2008), anti-cancerous (Mishra et al., 2008), dermal wound healing potential (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.
- Micropropgation of *R. imbricata* has not been done yet.

R. imbricata carries the plethora of pharmaceutical significance due to which the collection is reckless from its natural habitat. The plant can be propagated by using seeds and rootstocks cuttings for its multiplication under natural habitat (Chaurasia et al., 2007), but genetic uniformity cannot be maintained by these ex vitro techniques. So, in order to get clones of high content lines or shortening their juvenile phases, alternative techniques are needed to be employed for its mass multiplication.

To best of our knowledge, no micropropagation work has been done on *R. imbricata* till date. Therefore, the following objectives have been placed under studies.

- Optimization of culture conditions for callus induction and regeneration of plantlets.
- Optimization of conditions for direct shoot organogenesis for development of clones for multiplication and conservation.

CHAPTER - 2

REVIEW OF LITERATURE

Taxonomy and botany of *R. imbricata*

Around, 80% of the inhabitants in developing countries depend mostly on traditional medicine for health care needs, of which a bulk portion involves the utilization of plant extracts or their bioactive principles. One of the disparagements of herbal medicine is requirement of standardization and quality control profiles for the accurate recognition of the species concerned, whether in the fresh, dried or powdered state (Tayade 2015). The misclassification of species and the erroneous changeover is a real threat in the preparation and administration of herbal medicine (Tayade 2015). Some herbs are so strikingly similar in appearance to the untrained eye that they are normally mistaken for one another. 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* the information below is quite useful.

Cultivation and propagation of *R. imbricata*

According to cultivation experiences, *Rhodiola* spp. can be successfully grown for its root yield in South Finland using organic growing methods (Tayade 2015). For seedling production, seeds have to be sown in autumn, for natural winter stratification. The seedlings should be kept in pots for one year before transplantation, since the growth of the plants during the first 2-3 years is quite slow. The first root yield is harvested after four years from sowing and the root weight and root yield strongly 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).

Work done on other *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) containing recommended 3:1 ratio of Rosavins 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 *R. rosea* in the former Soviet Union. It has been categorized as adaptogen by Russian researchers. (Kelly 2001).

Tasheva et al. (2010) have developed efficient schemes for the regeneration and micropropagation of *R. rosea*. Monika et al. (2005) showed successful encapsulation of axillary buds and differentiating callus 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*.

Biosynthesis of 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 shown that salidroside possesses distinct functions such as anti-anoxic, anti-cold, anti-fatigue, anti-microwave radiation, anti-virus and anti-tumor, and also medicinal properties such as strengthening attention spans, improving work efficiency, delaying senility and preventing illness associated with old age. Due to its environmental acclimation activity, it plays important roles in military, aerospace, sports and healthcare.

The salidroside precursor p-tyrosol is a small phenolic molecule whose biosynthetic pathway and regulation are not completely understood. There are two different views on salidroside production in the literature. The first holds that tyrosol is produced from a p-coumaric acid precursor by a decarboxylase derived mainly from phenylalanine. The second states that the precursor of tyrosol may be tyramine synthesized from tyrosine (Zhang et al., 2011). Glycosylation of tyrosol is thought to be the final step in salidroside biosynthesis (Xu et al., 1998a). However, the efficiency of this reaction depends on tyrosol glucosyltransferase (*TGase*) activity. There are a few literature reports (Xu et al., 1998b; Yu et al., 2011) discussing the activity of *TGase* in the biotransformation process in *Rhodiola* spp. These investigations were concerned mainly with *R. sachalinensis*. Xu et al. (1998b) suggested that low yield of salidroside in *R. sachalinensis* cell culture was due to the low efficiency of glycosylation and a lack of synchronization between *TGase* activity and tyrosol accumulation in

the cells. Ma et al. (2007) showed that conversion of tyrosol aglycone to salidroside in *R. sachalinensis* could be regulated by *UGT73B6*. Overexpression of the *UGT73B* gene resulted in increased salidroside content in transformed *R. sachalinensis* plants (2-fold increase over untransformed control) and transformed calli (2.4fold increase). Yu et al. (2011) achieved isolation of two other uridine diphosphate (UDP)-glucosyltransferase cDNAs: *UGT72B1*4 and *UGT74R1*. The first transcript was more abundant in roots and the second was highly expressed in calli of *R. sachalinensis*. *UGT72B14* gave the highest salidroside production in vitro and in vivo. Transformed root cultures of *R. sachalinensis* have been studied as a potential source of salidroside (Zhou et al., 2007). Transformed root cultures were established by transformation with Agrobacterium rhizogenes and cultivated on liquid MS medium. Medium supplementation with tyrosol resulted in increased biomass and salidroside accumulation. A putative *TyrDC* gene was isolated from *R. rosea,* of which expression level was consistent with accumulation of salidroside. *TyrDC* as a key role in tyramine-derived compounds was supported by the study of overexpression of parsley *TyrDC* in potato leading to higher tyramine-derived compounds in potato [8]. In the present study, a full-length cDNA encoding *TyrDC* was cloned and functionally identified by feeding intermediate to recombinant protein from Tibet-specific *R. crenulata*; gene expression profiles were analysed in different tissues and in hairy root cultures with the treatment of different elicitors; the accumulation patter of

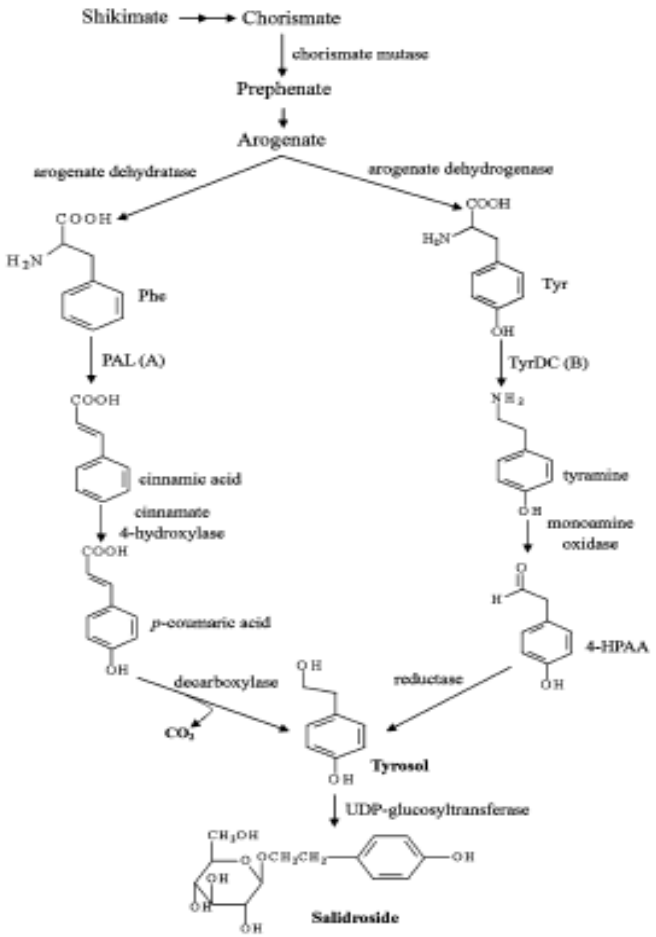


Figure 2. Proposed biosynthetic pathway of salidroside formation (Ma et al. 2008). Phe, phenylalanine; Tyr,; PAL, phenylalanine ammonialyase; TyrDC, tyrosine decarboxylase; 4-HPAA, 4-hydroxyphenylacetaldehyde

salidroside was also investigated; and finally based on the discoveries above, metabolic engineering strategy of overexpressing *RcTyrDC* was used to engineering sa lidroside biosynthetic pathway in hairy root cultures of *R. crenulata* that definitively improved biosynthesis of tyramine, tyrosol and the end-product salidroside.

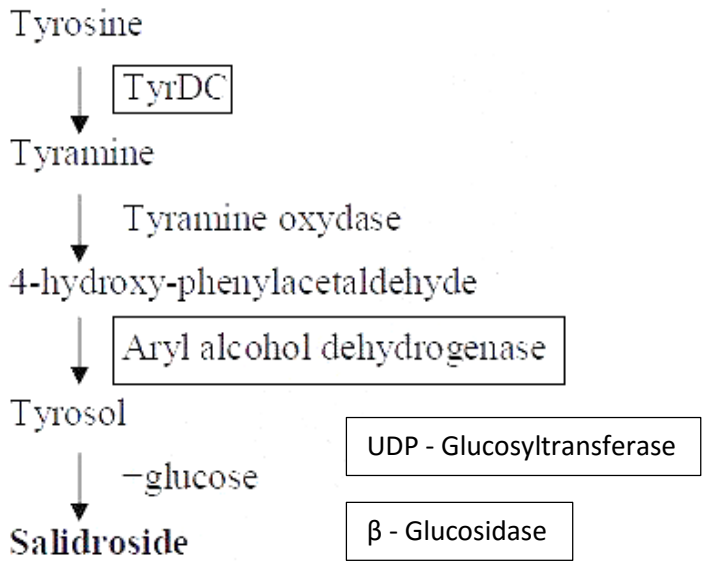


Figure 3. Biosynthetic pathway of salidroside synthesis in *Rhodiola rosea,* enzymes in boxes were targeted for primer designing

Biosynthesis of Rosavin

Cinnamoylglycosides like rosavins are the phenylpropanoids typically found in *Rhodiola* spp. These were first isolated, identified and designated by Zapesochnaya and Kurkin (1982). Rosavin is known as a stimulator of 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 highly significantly decreased neovascular reaction induced in the skin of Balb/c mice after grafting of L-1 sarcoma cells (Bany et al., 2008)

Both salidroside and cinnamyl alcohol glycosides are products of phenylpropanoid metabolism, derived from phenylalanine, which is a derivative of the shikimic-chorismic acid pathway. The enzyme that directs carbon to the synthesis of phenylpropanoid metabolites is known as phenylalanine ammonia lyase (*PAL*). *PAL* is the most extensively

studied enzyme in the phenylpropanoid pathway, if not in all secondary metabolism. It converts phenylalanine to cinnamic acid. At this point the pathway leaves the main phenylpropanoid biosynthesis way, which would lead to coumarins, flavonoids or lignins and lignans. However, the same types of enzymes take part in the further biosynthesis of the cinnamyl alcohol glycosides. From cinnamic acid cinnamyl CoA ester is formed through hydroxycinnamate:CoA ligase (*4CL*). This CoA ester is reduced to cinnamaldehyde by cinnamyl-CoA reductase (*CCR*). The cinnamaldehyde is further reduced by cinnamyl alcohol dehydrogenase (*CAD*) to cinnamyl alcohol. The enzymes that take part in the formation of the glycosides of cinnamyl alcohol are not yet described. By one glucose transfer rosin is formed, which is the simplest glycoside of roseroot. From rosin by the connection of an arabinose rosavin and by the connection of an arabinofuranose rosarin is formed. Depending on the sugar type and the site it is connected to, further glycosides may be formed.

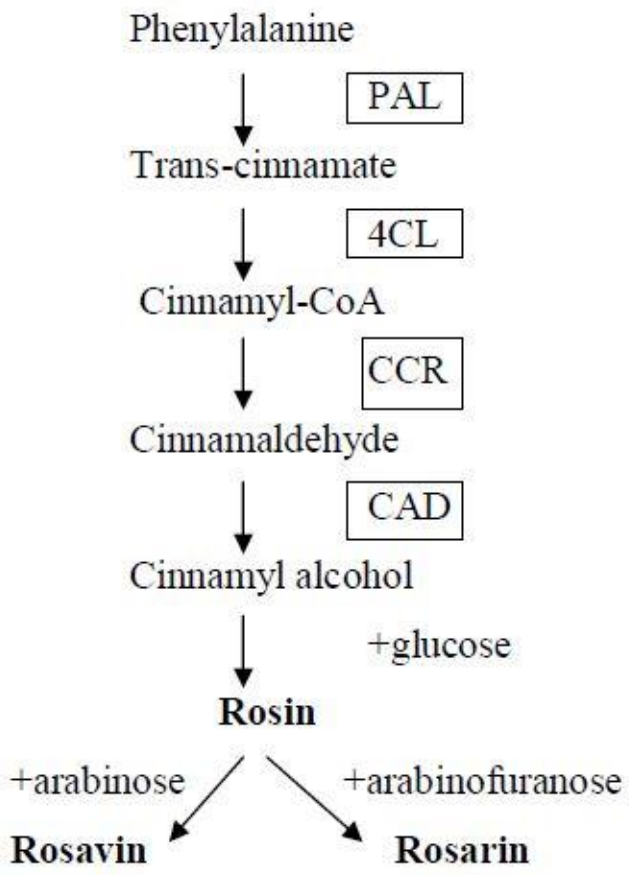


Figure 4. Biosynthetic pathway of rosavin and rosarin synthesis in *R. rosea*, enzymes in boxes were targeted for primer designing

In a study, *R. rosea* hydroalcohol extract (containing 3% rosavin and 1% salidroside) induced antidepressant-like, adaptogenic, anxiolytic-like and stimulating effects in mice (Perfumi et al., 2007).

Pharmacological properties of *R. imbricata*

In a study (Senthilkumar et al., 2014), hepatoprotective activity of *R. imbricata* rhizome acetone extract against paracetamol (2 g/kg) induced liver toxicity was estimated. Paracetamol was administered to induce hepatic damage in Wistar rats. 200 and 400 mg/kg doses of rhizome acetone extract and silymarin (25 mg/kg) were used as treatment groups. The blood samples were analyzed for biochemical markers of hepatic injury and tissue samples were subjected for estimation of liver antioxidants and histopathological studies. Analysis of the extract treated rats (400 mg/kg) showed an elevation of superoxide dismutase (0.326 units/min/mg protein), catalase (185.03 µmole of H_2O_2 consumed/min/mg protein), glutothione peroxidase (19.26 mg GSH consumed/min/mg protein) and reduced glutathione (16.2 lmole of GSH/mg protein). Moreover, the biochemical parameters in serum like alkaline phosphatase, serum glutamic oxaloacetic transaminase (*SGOT*), serum glutamic pyruvic transaminase (*SGPT*) and lipid profiles were also improved in treated groups compared to the control. The oral administration of different doses of rhizome acetone extract significantly protected the hepatic cells from damage. The haematological and biochemical parameters were also normal in extract treated rats compared to the control and standard (silymarin) groups. The HPLC analysis revealed the presence of some important phenolic compounds which could be responsible for the hepatoprotective activity. This study proved that *R. imbricata* could be taken as a good natural source of the hepatoprotective agent.

Cytoprotective and antioxidant activity of aqueous and alcoholic extracts of *R. imbricata* rhizome on tert-butyl hydroperoxide (tert-BHP) induced cytotoxicity in U-937 human macrophages was also reported (Kanupriya Prasad et al., 2005). There was an increase in cytotoxicity and apoptosis significantly in the presence of tert-BHP over control cells. The tert-BHP induced cytotoxicity can be attributed to enhanced reactive oxygen species (ROS) production which in turn is responsible for fall in reduced glutathione (GSH) levels; further there was a significant decrease in mitochondrial potential and increase in apoptosis and DNA fragmentation. Both aqueous and alcoholic extracts of *Rhodiola* rhizome at a concentration of 250 microg/ml were found to inhibit tert-BHP induced free radical production, apoptosis and to restore the anti-oxidant levels to that of the control cells. The alcoholic extract of *Rhodiola* showed higher cytoprotective activities than aqueous extract. These observations suggest that the alcoholic and aqueous extracts of *Rhodiola* have marked cytoprotective and antioxidant activities.

The wounds treated with ethanolic extract of *R. imbricata* rhizome were found to heal much faster as indicated by the improved rate of wound contraction and decreased time taken for epithelialization (Gupta et al., 2007). The extract treatment increased cellular proliferation and collagen synthesis at the wound site, as evidenced by the increases in DNA, protein, hydroxyproline and hexosamine contents in comparison to a positive control treated with povidone-iodine ointment. These results were also supported by histological examinations. Furthermore, the treatment also caused an in-crease in antioxidant and a decrease in lipid peroxide levels in the granulation tissue. The results suggest that *R. imbricata* rhizome ethanol extract possesses a significant wound healing activity.

Immunomodulatory activity of aqueous extract of *Rhodiola* rhizome in human peripheral blood mononuclear cells (PBMCs) and mouse macrophage cell line RAW 264.7 was also evaluated (Mishra et al., 2006). The *Rhodiola* extract was found to stimulate production of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in human PBMCs as well as RAW 264.7 cell line. It also increased production of nitric oxide synergistically in combination with lipopolysaccharide (LPS) in RAW 264.7. *Rhodiola* at 250 μg/ml increased the p-IκB expression in human PBMCs. Aqueous extract of *Rhodiola* (250 μg/ml) also activated the nuclear translocation of NF-κB in human PBMCs, which is comparable to the positive stimulant LPS. This study suggested that *Rhodiola* most likely activates proinflammatory mediators via phosphorylated inhibitory kB and transcription factor NF-kB and also demonstrated immunostimulatory potential of aqueous extract of *Rhodiola* rhizome that can be used for upregulation of immune response in patients with inadequate functioning of the immune system.

Aqueous extract of *R. imbricata* root possess potent adaptogenic activity with no acute and sub-acute toxicity (Gupta et al., 2008). To examine this, aqueous extract of *R. imbricata* root was orally administered in rats at different doses, 30 min prior to cold (5 °C)–hypoxia (428 mm Hg)–restraint (C–H–R) exposure. The maximal effective adaptogenic dose of the extract was 100 mg/kg body weight. The acute and sub-acute toxicity of the extract was also studied in rats. Sub-acute toxicity studies included administration of single oral dose of 1 g/kg and 2 g/kg of extract once daily for 14 days and maximal effective single oral dose of 100 mg/kg once daily for 30 days. At the end of each treatment period the biochemical parameters related to liver function, kidney function, lipids (triglycerides, cholesterol) and hematological parameters were estimated in serum and blood. Biochemical and hematological analysis showed no significant changes in any of the parameters examined in treated group's animal, in comparison to control animals. No significant change was observed in organ weight/body weight ratios, of any organ studied in comparison to control rats. The

oral LD_{50} of the extract was observed to be >10 g/kg, indicating an adequate margin of safety. No histopathological changes were observed in the vital organs studied of the treated animals.

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

The anti-proliferative effects of *Rhodiola* aqueous extract (RAE), were also studied in human erythroleukemic cell line K-562 using MTT cell proliferation assay (Mishra et al., 2008). The proliferation of K-562 was significantly decreased after 72 h incubation with RAE at 100 and 200 μg/ml. However, almost no suppressive effects could be detected in normal human peripheral blood lymphocytes or mouse macrophage cell line RAW-264.7. RAE was also found to induce intracellular reactive oxygen species (ROS) in K-562 cells at 200 μg/ml when incubated overnight. The increased ROS generation may cause apoptosis, which was observed in AnnexinV-FITC and propidium iodide (PI) staining of cells treated with RAE for 72 h in K-562 cells. Moreover, RAE arrested cell cycle progression in G2/M phase in early and late period of exposure. The anti-cancer activity of RAE was also confirmed by increased NK cell cytotoxicity. These observations suggest that aqueous extract of *R. imbricata* rhizome has very potent anti-cancer activities, which might be useful in leukemia cancer treatment.

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*.

CHAPTER - 3

MATERIALS

AND METHODS

Selection of plant material

The *R. imbricata* plants were procured from the Defence Institute of High Altitude Research (DIHAR), DRDO, Ministry of Defence, Leh, India and were maintained in greenhouse of Jaypee University of Information Technology, Waknaghat, H.P., India at 25 ± 2°C, 80–90 % of relative humidity with light irradiance of 100–120 µmol m^{-2} s^{-1} under natural light conditions (Figure 5).

Figure 5. *R. imbricata* planted in greenhouse of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India.

Media preparation and culture conditions

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. The pH of the each media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. All the media were autoclaved at 121°C and 15 lb/in pressure for 15 - 20 min. The cultures were incubated in two different plant tissue culture chambers maintained at 15 ± 2°C and 25 ± 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).

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)

Selection and culture of explants

Explants such as leaf, internodal segments and root were taken from plantlets of *R. imbricata* grown in greenhouse of Jaypee University of Information Technology, Waknaghat, H.P., India. These explants were thoroughly washed with few drops of Labolene and were surface sterilized in 0.5% Bavistin and 0.1% mercuric chloride followed by 4 - 5 washings in sterile water under aseptic conditions.

Callus initiation

These sterilized explants were cultured for callus induction on above mentioned media (Table 1) while giving injuries to the explants while culturing in order to initiate callus formation. The cultures were incubated under above mentioned culture conditions. Data on days to initiation of callus formation, callus morphology and percentage of calli were recorded. These experiments were

performed in triplicates and repeated thrice. The cultures were sub-cultured after every 15 – 20 days on callus induction media for 2 months so as to obtain good growth.

Plantlet regeneration from calli

After the explants were completely transformed into callus mass, the calli or parts thereof were transferred onto media consisting of MS salts supplemented with different concentrations of IBA, KN, BAP, GA_3 and TDZ (Table. 1) under aseptic conditions for differentiating into shoots and incubated in the plant tissue culture chamber maintained at 15 ± 2°C and 25 ± 2°C temperatures. The data on days to shoot regeneration, average shoot numbers and shoot length were recorded. These experiments were performed in triplicates and repeated thrice.

Direct shoot organogenesis and multiplication

Shoot apex and lateral shoot buds were selected as explant for direct shoot organogenesis by following the same culture procedures as mentioned above. The proliferated shoots from the buds were further multiplied on the above mentioned media from their mass multiplication and for root induction. Similarly, regenerated shoots from callus were excised and transferred into media consisting of MS salts supplemented with different concentrations of IBA, KN, BAP, GA_3 and TDZ (Table. 1) under aseptic conditions for multiplication of shoots and incubated in the plant tissue culture chamber maintained 15 ± 2°C and 25 ± 2°C temperatures. The data on days to shoot multiplication, number of shoots and average shoot length were recorded. These experiments were performed in triplicates and repeated thrice.

Rooting

Individual shoots were taken and transferred on media consisting of MS salts supplemented with different concentrations of IBA, KN, BAP, GA_3 and TDZ (Table. 1) under aseptic conditions for rooting of shoots and incubated in the plant tissue culture chambers maintained at 15 ± 2°. The data on days to root initiation, number of roots per shoot, average root length and percent shoot forming roots were recorded. These experiments were performed in triplicates and repeated thrice.

Hardening

The rooted shoots were gently removed from the culture vessels, washed under running tap water and transferred to pots containing sand: soil: vermiculite (1: 1: 1) in the greenhouse conditions for acclimatization and hardening. The plantlets were covered with glass jars for 10–15 days to avoid desiccation. Glass jars were taken off every day for 1–2 h so as to acclimatize them to external environment. Data was recorded for percent survival of plants.

CHAPTER - 4

RESULTS AND DISCUSSION

Establishment of callus cultures

Callus cultures were initiated from leaf, intermodal segment and root explants of *R. imbricata* on different media combinations (Table. 1). Out of tested 8 media combinations, callus was initiated in 6 media combination *viz.* RI2, RI3, RI4, RSI, RI6 and RI7 at 15 ± 2°C and 25± 2°C. Callus initiation was observed earlier at 15 ± 2°C (10-55 days) compared to 25± 2°C (20 -70 days) at the cut surfaces (Graph). MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l) was found to be best with callus induction in 10 - 35 days with 71 - 92% of calli percentage from leaf, intermodal segments and root explants at 15 ± 2°C (Table 2). Sharif et al. (2016) also showed that MS media supplemented with 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). Leaf explant was found to be best for callus induction in 10-15 days with 92% of calli percentage (Figure 6). Leaf explants were transformed into complete callus mass within 4 weeks of culture. The callus mass was maintained by subculturing on RI3 after 4 -5 weeks. Calli with a green and creamy appearance were taken for regeneration.

Regeneration of shoots from calli

Proliferating callus cultures or parts thereof were subcultured onto regeneration media containing MS salts supplemented with different media combinations (Table.1). Out of tested 8 media combinations, callus was regenerated into shoots in 3 media combination *viz.* RI1, RI3, and RI7 at 15 ± 2°C and 25± 2°C. Shoot regeneration was observed earlier at 15 ± 2°C (20-25 days) compared to 25± 2°C (30-40 days). Similarly, *Picrorhiza kurroa* plants were found to be healthier at 15 ± 2°C compared to 25± 2°C due to accumulation of hemicellulose at low temperature (Kawoosa et al., 2010). MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l) was found to be best with shoot regeneration in 20 - 25 days with 15.23±0.05 shoot number and average shoot length of 2.50±0.03 at 15 ± 2°C.(Table 3)(Figure 7). The same medium combination was found most suitable for obtaining maximum callus and the highest number of shoots/callus from calli.

Table 2. Effect of MS + auxins + cytokinins + defoliants on callus induction from explants of *R. imbricata*.

		Leaf explant			Internodal segments			Root explant		
S.No.	**Medium name**	Days for callus initiation	Callus color	Percent calli (%)	Days for callus initiation	Callus color	Percent calli (%)	Days for callus initiation	Callus color	Percent calli (%)
1.	RI0	-----	-----	-----	-----	-----	-----	-----	-----	-----
2.	RI1	-----	-----	-----	-----	-----	-----	`-----	-----	-----
3.	RI2	15-20	Cream	79%	35-40	Cream	46%	45-50	Brow	12%
4.	RI3	10-15	Green	92%	30-35	Green	71%	30-35	Green	20%
5.	RI4	15-20	Green	60%	37-42	Cream	52%	50-55	Brow	15%
6.	RI5	25-30	Cream	62%	35-40	Cream	57%	30-35	Brow	9%
7.	RI6	25-30	Green	74%	32-37	Green	62%	40-45	Green	18%
8.	RI7	12-17	Green	68%	32-37	Green	59%	35-40	Brow	16%
9.	RI8	-----	-----	-----	-----	-----	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----	-----	-----	-----	-----	-----

Table 3. Effect of MS media on shoot regeneration from calli of *R. imbricata*.

S.No.	Medium name	Days for shoot regeneration	Average no. of shoots	Average shoot length (cm)
1.	RI0	-----	-----	-----
2.	RI1	25-30	9.52±0.05	2.23±0.02
3.	RI2	-----	-----	-----
4.	RI3	20-25	15.23±0.05	2.50±0.03
5.	RI4	-----	-----	-----
6.	RI5	-----	-----	-----
7.	RI6	-----	-----	-----
8.	RI7	30-35	7.34±0.07	1.72±0.01
9.	RI8	-----	-----	-----
10.	RI9	-----	-----	-----

*Average = Mean±SD

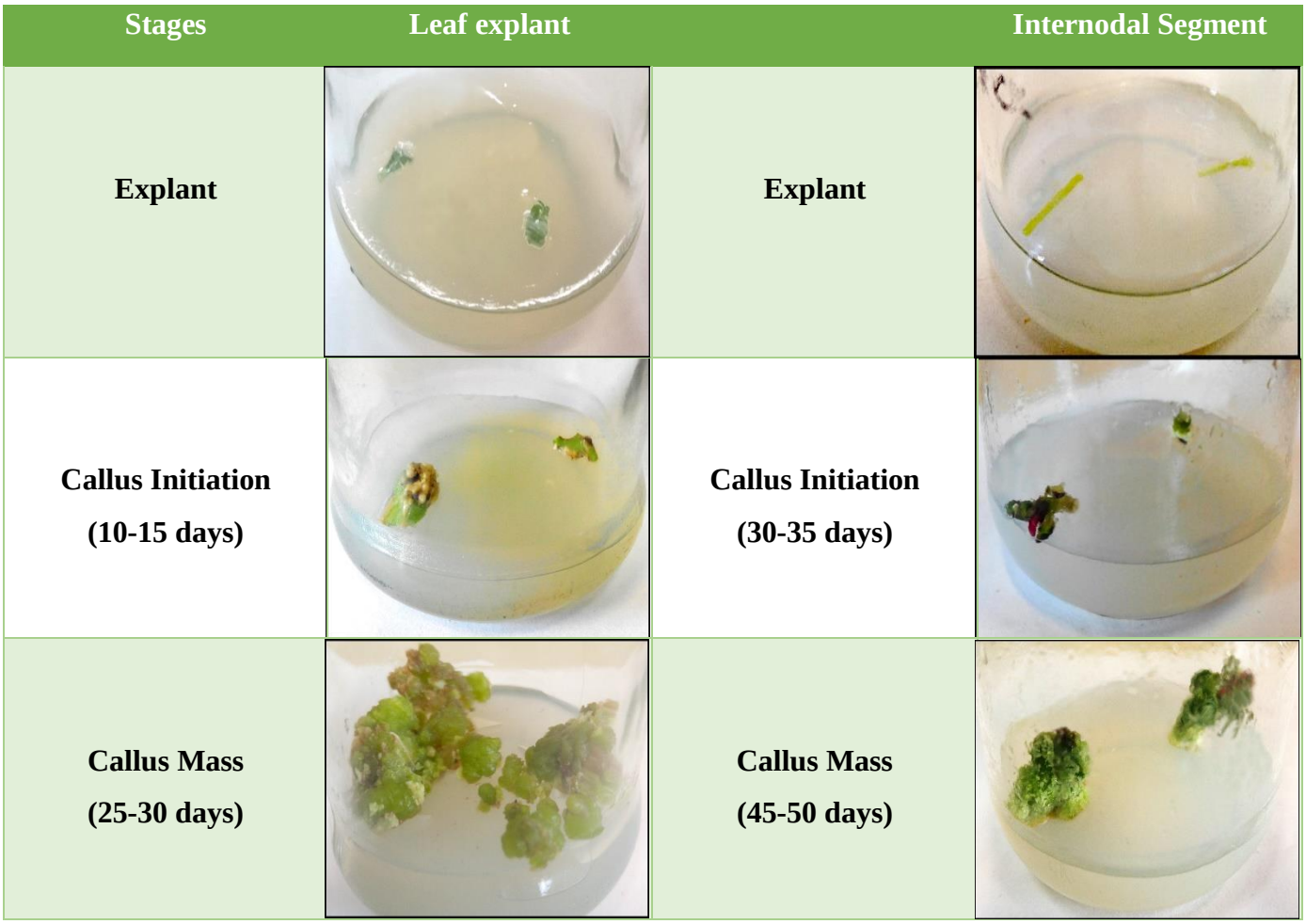


Figure 6. Callus initiation and proliferation from different explants of *R. imbricata*.

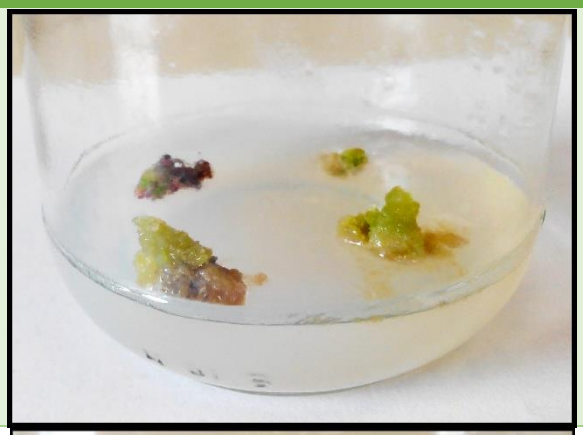
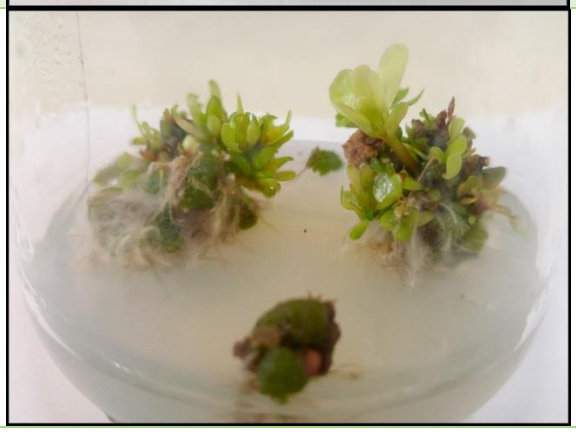
Stages	Shoot Regeneration
Emergence of shoot primordia (10 days)	
Differentiating primordia (15 days)	
Shoot regeneration (20-25)	
Shoot elongation and growth (30-35)	

Figure 7. Regeneration of callus cultures into shoots in *R. imbricata.*

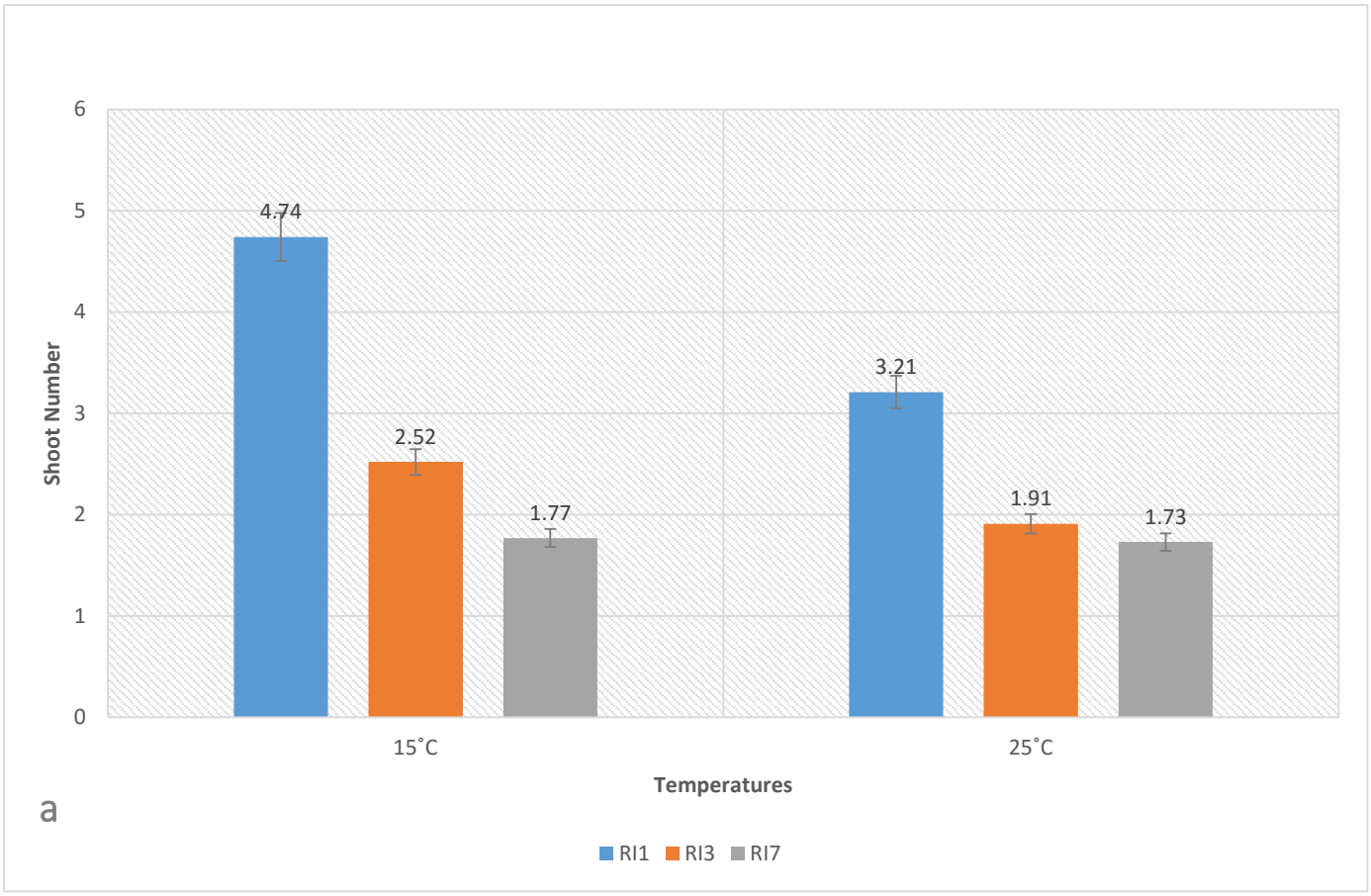
Direct shoot organogenesis and multiplication

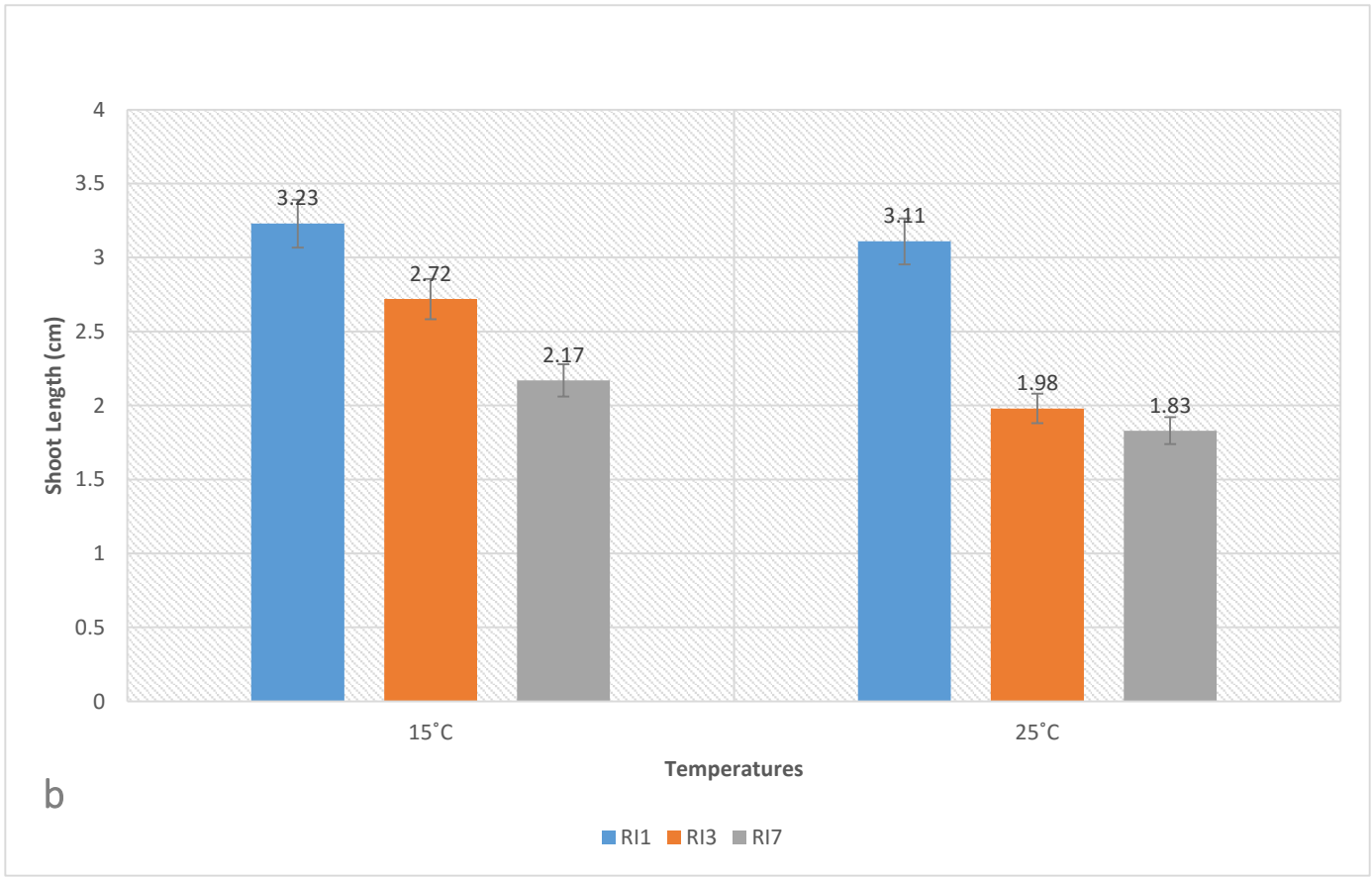
Shoot apex and lateral shoot buds taken for direct shoot organogenesis and regenerated shoots from callus were subcultured on different media combination (Table. 1). Out of tested 8 media combinations, shoot was multiplicated in 3 media combination *viz.* RI1, RI3, and RI7 at 15 ± 2°C and 25± 2°C. Shoot multiplication was observed earlier at 15 ± 2°C (18-23 days) compared to 25± 2°C (25-30 days). Also, shoot number and shoot length was found to be more in 15 ± 2°C (Graph 1). Similarly, Sharma et al., (2016) have shown better growth with high secondary metabolite content in *Picrorhiza kurroa* plants at 15 ± 2°C compared to 25± 2° C. The MS medium supplemented with 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±0.02 shoot number and average shoot length of 3.23±0.03 at 15±2°C (Table 4) (Figure 8).

Table 4. Effect of various media on shoot multiplication of shoots derived after regeneration from callus of *R. imbricata*.

S.No,	Medium name	Days for shoot multiplication	Average no. of shoots	Average shoot length (cm)
1.	RI0	-----	-----	-----
2.	RI1	18-23	4.74±0.02	3.23±0.03
3.	RI2	-----	-----	-----
4.	RI3	16-20	2.52±0.01	2.72±0.02
5.	RI4	-----	-----	-----
6.	RI5	-----	-----	-----
7.	RI6	-----	-----	-----
8.	RI7	20-25	1.77±0.03	2.17±0.07
9.	RI8	-----	-----	-----
10.	RI9	-----	-----	-----

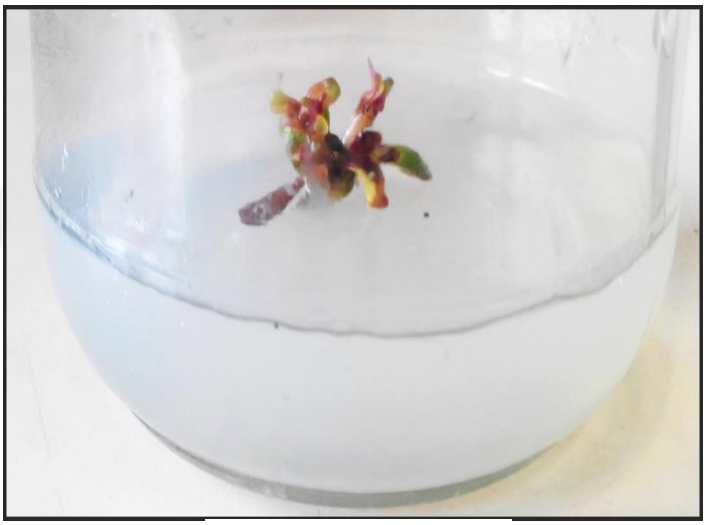
*Average = Mean±SD





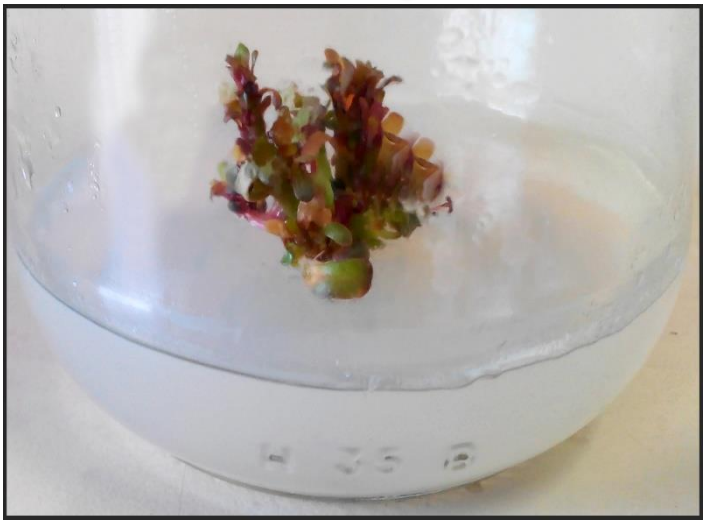
Graph 1. Difference in (a) Shoot number (b) Shoot length w.r.t. temperatures.

5 days



10 days

15 days



20 days

Figure 8. Shoot multiplication of *R. imbricata.*

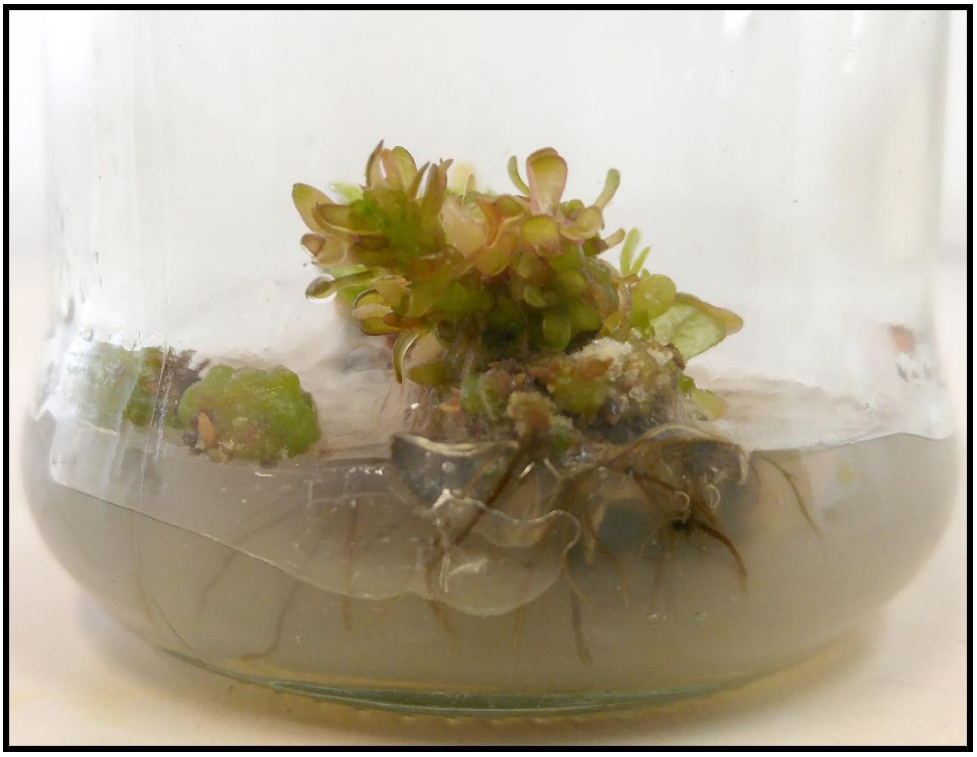
Rooting and Hardening

Shoots were cultured on different media combinations (Table. 1) for root induction. Out of tested 8 media combinations, rooting was obtained in 3 media combination *viz.*RI3, RI6 and RI7 at 15 ± 2°C. The MS medium supplemented with BAP (2 mg/l) + IBA (4 mg/l) was found to be the best for rooting in 25-30 days with 4.4±0.05 root number, average root length of 5.6±0.03and 71% of shoot forming roots at 15±2°C (Table 5) (Figure 9). Sharif et al. (2015) also showed that MS media supplemented with IBA and BAP produce root proliferation from broccoli using root tip cultures. In vitro rooted plantlets showed survival rate of 75 - 80 % after hardening in greenhouse conditions (Figure 10).

Table 5. Effect of various media on rooting of shoots derived after regeneration from callus of *R. imbricata*.

*Average = Mean±SD

S.No.	Medium name	Days for root initiation	Average no. of roots/shoot	Average root length (cm)	Percent shoot forming roots (%)
1.	RI0	-----	-----	-----	
2.	RI1	-----	-----	-----	
3.	RI2	-----	-----	-----	
4.	RI3	35-40	4.1±0.01	5.2±0.06	63%
5.	RI4	-----	-----	-----	
6.	RI5	-----	-----	-----	
7.	RI6	25-30	4.4±0.05	5.6±0.03	71%
8.	RI7	40-45	3.9±0.04	4.7±0.02	42%
9.	RI8	-----	-----	-----	-----
10.	RI9	-----	-----	-----	-----



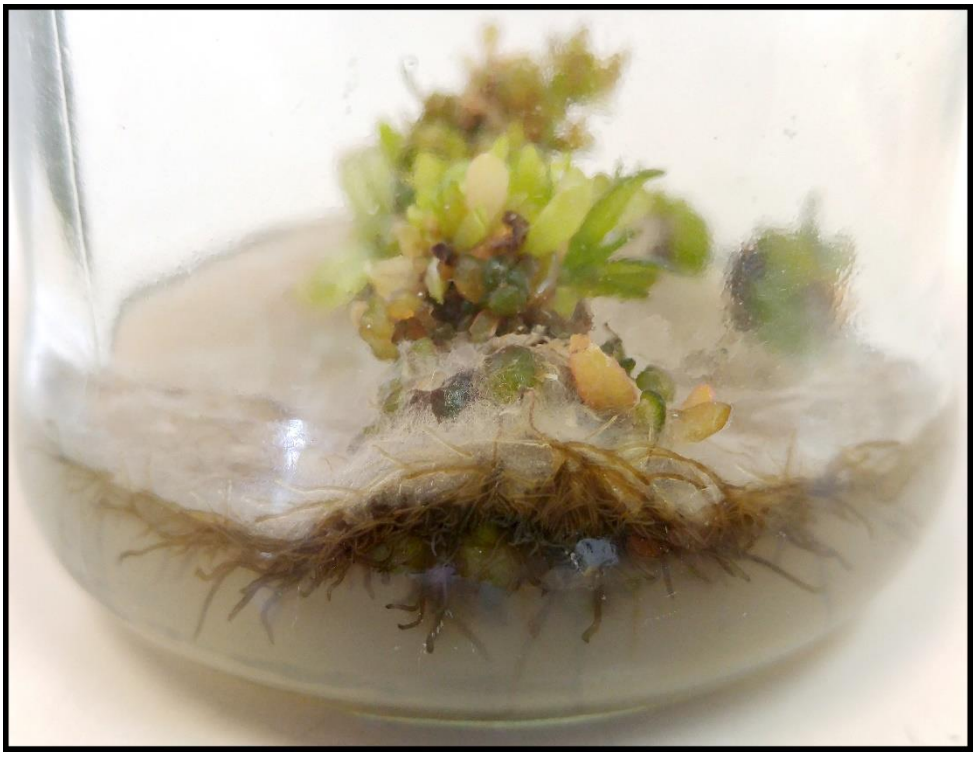


Figure 9. Rooting of multiplicated shoots of *R. imbricata.*

Figure 10. Hardening of *R. imbricata.*

The overall objective of the current study was to develop an *in vitro* system for micropropagation from explants of *R. imbricata*. There are reports in medicinal and aromatic plants wherein the metabolites of medicinal importance 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 *R. imbricata* has been declared as an endangered medicinal herb which warrants that tissue culture conditions need to be standardized for the plant so that the cultures can be used for the production of metabolites under laboratory conditions as has been reported in various medicinal plants so as to relieve pressure from its natural habitat (Vanisree et al., 2004). Also due to the presence of various 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. For this, pharmaceutical industries will be requiring large biomass which can only be met by adopting tissue culture techniques.

The nutrient media forms the main component for induction of callusing and plant regeneration in tissue cultures. The major differences in the response of different plant species and different explants to tissue culture conditions lies in the ratio of auxins to cytokinins (Skoog and Miller, 1957). Shoots were also regenerated from callus in regeneration media containing different concentrations and combinations of auxins and cytokinins. Hence a complete protocol for callus induction and shoot regeneration has been standardized for *R. imbricata*. The protocol is expected to be of immense practical importance in selection and regeneration of cell lines of *R. imbricata* with enhanced content of medicinally important phytopharmaceuticals. The conditions of callusing and regeneration can also be used in genetic transformation of *R. imbricata* for increased phytopharmaceuticals production through metabolic engineering by using Agrobacterium-mediated transformation. Being endangered in its natural habitat, the cell/callus cultures can be cryopreserved for its sustainable conservation (Grout, 2007). The protocol can be further used to meet high demand of raw material worldwide for production of pharmaceutical products from *Rhodiola*.

CHAPTER - 5

CONCLUSION AND FUTURE WORK

R. imbricata which 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 culture conditions for its mass multiplication and production of in vitro grown plantlets.

In our present study, we have tried various growth hormone combinations for callus induction, shoot regeneration from callus, shoot multiplication and root induction for *R. imbricata*

- MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l) was found to be the best for callus induction as well as shoot regeneration from callus.
- MS medium supplemented with BAP (1 mg/l) + KN (2 mg/l) was found to be the best for the shoot multiplication.
- MS medium supplemented with BAP (2 mg/l) + IBA (4 mg/l) was found to be the best for root initiation in *R. imbricata.*
- After hardening in greenhouse conditions, in vitro rooted plantlets showed survival rate of 75 - 80 %.

Thus, the developed technology would be utilized further for the important secondary metabolite production and also for the elicitation of those compounds under optimized culture conditions for their large scale production in order to meet the demand of herbal and pharmaceutical industries.

LIST OF PUBLICATIONS

- **Shubham Sharma**, Sahil Kapoor, Neha Sharma, Rajinder Singh Chauhan, O. P Chaurasia and Hemant Sood (2016). **Callus induction and shoot regeneration for conservation of high value medicinal plant *Rhodiola imbricata***. 3rd International Conference on Biotechnology and Bioinformatics (ICBB-2016). [5-7 Feb, 2016 Pune, India,]

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