Expression analysis of key genes for medicinal compounds production and biosynthesis in tissue cultures of *Rhodiola imbricata.*

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WAKNAGHAT

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DECLARATION

I ensure 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 - Chetansee Khanna

Enrollment No. - 133815

Date -

ACKNOWLEDGEMENT

Each task huge or little is fruitful to a great extent because of the exertion of various magnificent individuals who have constantly given their significant counsel or loaned some assistance. I earnestly value the motivation; support and direction of each one of those individuals who have been instrumental in making this undertaking a win.

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At this crossroads I feel profoundly regarded in communicating my true because of Dr. Hemant Sood for making the assets accessible at ideal time and giving significant bits of knowledge prompting the effective finishing of my undertaking.

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Last however not the slightest I put a profound feeling of appreciation to my relatives and my companions who have been steady wellspring of motivation amid the arrangement of this task work.

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SUMMARY

Rhodiola imbricata Edgew. is a lasting herb of the family Crassulaceae, found in extraordinary Himalayan area at a height of 4000– 5000 m. It is additionally accessible in fringe districts of Leh – Ladakh, China and Tibet. R. imbricata has been utilized broadly for its therapeutic properties in conventional people prescription in China, Tibet, Mongolia and the previous Soviet Republics to increment physical perseverance, work efficiency, life span and to treat weariness, asthma, discharge, barrenness and gastrointestinal infirmities. It additionally have different pharmacological exercises like hepatoprotective, radioprotective, cytoprotective, injury recuperating, Immunomodulatory, hostile to weakness, neuroprotective, anticancerous and so on., which is because of the nearness of different phytochemicals, for example, p-tyrosol, Salidroside, Rosavin, gallic corrosive, and so on. To the best of our insight, this is the principal provide details regarding sub-atomic portrayal of Rhodiola imbricata for biosynthesis of Salidroside and Rosavin. In this investigation, differentiating conditions were additionally created utilizing MS media and distinctive development hormones blends and diverse temperatures for the collection of auxiliary metabolites and for transcriptome age. It was discovered that at 15±2 C° and 25±2 C°, MS medium brought about the greatest generation in callus, though shoots kept up at 15±2 C° and 25±2 C°, media having BAP (1 mg/l) + KN (2 mg/l) brought about best development. Qualities in charge of the biosynthesis of Salidroside and Rosavin in callus and in vitro shoots were distinguished which are TyrDC, UDPGT, AAD and BGD and their successions were likewise produced for examining articulation investigation for biosynthesis of rosavin and salidroside. So present examination gives roads to encourage investigations and examinations in trans-Himalayan herb Rhodiola imbricate for its pharmaceutical uses.

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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 NO.-1

INTRODUCTION

The The stone product family, Crassulaceae involves more than fourteen thousand species that are spread in thirty three classes which also comprises of Rhodiola and is regularly disseminated overall particularly in the Northern Side of the equator and South Africa. The depictions, circulation and gathering destinations of the different types of the family are accessible in various written works (1). This specific family develop in depleted lands at an elevation of twenty seven hundrem meters to five thousand meters and have existing from ages, to have monstrous restorative prospectives. This plant, which comprises of almost ninety species, are broadly circulated in elevated height cool abandon locale of the Northern Half of the globe. Numerous Rhodiola species have been utilized as a part of conventional medications for the treatment of long haul ailment and shortcoming because of contamination in Tibet and different areas for more than 1000 years (2).

India itself comprises of 6 types of Rhodiola are there, to be specific R. heterodonta, R. imbricata, R. quadrifida, R. sinuate, R. tibetica, and R. wallichiana (3). Rhodiola imbricata Edgew already known as Sedum roseum. It is a succulent perpetual herb privately known as rose root because of the rose-like smell (scent) of the new cut rootstock (4). Some of the used parts are roots as well as rhizomes which have properties like frost tolerancy, restorative properties and many adaptogenic characterstics (5).

R. imbricata Edgew. is a dioecious, herbaceous perpetual plant, starting in the mountain locales of South West China and local around entire of the Northern half of the globe (6). It is a moderate developing; lasting patio nursery plant that reaches out from the Pakistan, India, Nepal to China (6).

Rhodiola (Fig. 1) is a luscious plant and a bulky rhizome, brilliant appearance, pink color at inside of the plant, ten to thirty five centimeter with rose scented enormous rootstock; leaves 1.3-3 cm long, oval to limit elliptic(7).

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

Figure 1. The objective shrub *R. imbricata* found in trans-Himalayan cold desert of Ladakh region, India (Tayade 2015)

Some of its species are utilized for customary prescriptions for the dealing of extensive haul ailment as well as shortcomings because of disease in Tibet and different locales for more than 1000 years (8). R. imbricata is a critical conventional restorative plant and is generally utilized as sustenance edit and is appropriated in trans-Himalayan cool leave areas. In some countries arrangement of customary drug, all roots are utilized for treating lung issues, icy, hack, fever, vitality loss along with pneumonic protestations (9). Some of the other pharmacological properties of R. imbricata are immunomodulatory potential, immunostimulatory action (10), adjuvant action, adaptogenic movement, radioprotective adequacy, radiomodulatory, cytoprotective (11), cell reinforcement potential (12), free-radical searching action and metal chelating action (13), icy, hypoxia and restriction (C-H-R) introduction and post-push recuperation (14), icy, hypothermia actuated by pressure and recuperation after pressure(15): instrument and activity, against proliferative impacts, against dangerous, dermal injury mending potential (16), hepatoprotective impact, radical rummaging and antiproliferative action of concentrates in human colon and tumor cells (17), and observed to be sheltered (18).

In light of the previously mentioned progressing research, following exploration holes in R imbricata examine have been taken note.

- Although, different in vivo and in vitro pharmacological possibilities are all around archived in polar concentrates, the dynamic guideline of the plant in these concentrates still remains a fantasy (19).

- Moreover, the plant is broadly utilized as a consumable plant in India, Nepal, Tibet, and China, its nourishing quality regarding its vitamins, unsaturated fats, amino acids, and mineral substance still (20).

stayed uncharted in this plants root aside from the variety was tried just for having the substantial metals, for example, As, Pb, Hg, Album, Zn, Cu, and Cr (21).

• Unstable, semi-unpredictable and glacial mixes in various concentrates should be investigated to determine the medicinal capability of rhodiola (22).

• Presently continuous researches uncovered crucial medical activities of many the plant tried using various solvents. Be that as it may, the dynamic standards in these concentrate still stayed unrevealed (23).

Because of the nearness of specified pharmacological exercises, R. imbricata can be utilized by pharmaceutical businesses to create drugs to expand stamina, work profitability, life span and to diminish exhaustion, stress and melancholy (24). Along these lines thinking about, appeal of Rhodolia crude material worldwide for pharmacological use, event of species in unforgiving ice or uneven cool disserts, over abuse from regular populaces, characteristic troubles in development, this examination is projected to explore the likelihood of generation of bioactive mixes (25).

To best of our knowledge, no reports on expression analysis of genes involved in production of rosavin and salidroside in *R. imbricata* are present till now. Following are the objectives of my studies:.

- Optimization of contrasting conditions for salidroside accumulation in dissimilar in-vitro full-grown tissues of *Rhodiola imbricata*.
- Expression analysis of key genes which are responsible for accumulation and synthesis of Salidroside and Rosavin as secondary metabolites in *R. imbricata*.

CHAPTER NO.- 2

REVIEW OF LITERATURE

Scientific categorization and plant science of R. imbricata

Almost, eighty percent of the populace in making countries looks for the most part on regular medicines for human administrations wants, of which a crowd fragment incorporates the utilization of the plant evacuates or their therapeutic principles (26). Some of the defamations of self developed drug are essential of standardization and quality assurance consents for the exact affirmation for variety anxious about. A team of herbs are actually markedly alike in manifestations to the inexpert senses that all of them are frequently stimulated for everyone (27).

Development and spread

Reported by improvement documents, Rhodiola plants are viably produced for its root metabolites in many countries using common methodologies. Whereas, to grow seeds, it must be soaked before arrival of winters (28). For that these are placed in pots for 1 year from the time transplantation is done (29). One of the special yields are collected after completion of 4 years from starting of this step (30).

Improvement along with causing sharpeness show plant is cultivated using seeds as well as rootstocks cuttings. More than half of seed germination was proficient in open lands while if there ought to be any chance of root stocks farm eighty six percent of the endurance rate has been observed (31).
Some reports showed have made powerful plans for the recuperation and micropropagation of R. rosea. Some papers showed productive encapsulation of isolating callus and axillary buds in calcium alginate spots in R. kirilowii. These cases could frame into shoots and plantlets on solid MS basal medium (32).

Medicinal characterstics of *R. imbricata*

One of the examination (33), showed hepatoprotective activity of plants rhizome for treating disorders of liver impelled by usage of medicines (34). Furthermore, assessment of liver growth avoidance operators finished using tissue cultures and their tests, close by histopathological looks. Plus, difference in the biochemical parameters in serum like acid neutralizer phosphatase, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and lipid profiles were in like manner found in the treated social occasions diverged from the control(34).

Reports shows that wounds treated with these plants extracts, recovers faster and show proper healing (35). Also, when a cell support was developed for a decreasing lipid and its components profiles using the extracts, it showed good results, followed after any surgical treatment, which shows the plants damage repairing and recovery properties (36). Some papers reported about the adaptogenic quality of the plant especially against toxicity related profiles (37).

Primary secondary metabolites

- **Salidroside**

This metabolite guarantees erythrocytes derived from humans, of its cell strengthening activity among a measured secondary form. It also guard the liver and kidney cells from various oxidative stress by producing guarding proteins through various genes (38). Various experimentations and tests resulted in variety of its properties which includes protection against anoxic, aggressiveness to chilly, threatening of fatigue, unfriendly to contamination and unfriendly to cancer cells (39). Other reported properties are increase in working rate, decreasing weakness, and removing retardants of various forms of development (40).

Its precursor molecule is repoeted to be a phenolic molecule, known as p-tyrosol whose synthetic pathway is not much discussed or known yet (41). The two discussed pathways for its synthesis have different routes, one in which this phenol molecule attaches with p-coumaric molecule with the help of decarboxylase enzyme which is derived directly from phenylalanine (42). The other route is when this phenol molecule is actually a derivative of tyrosine (43). The last step of this pathway to be deciphered is glycolysis of tyrosol (44).

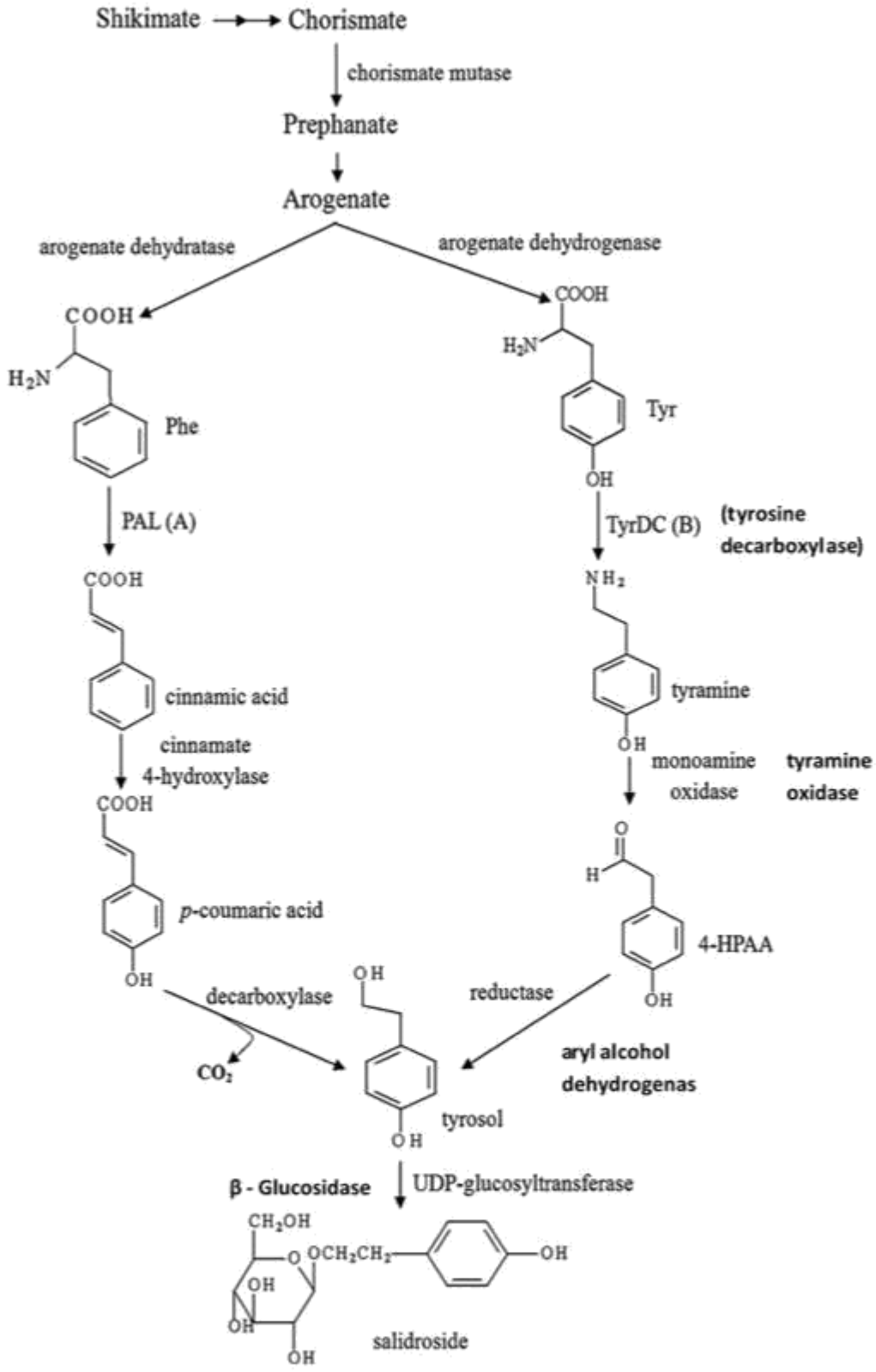


Fig 2. Devised pathway for salidroside synthesis.

When TGase does not works properly with tyrosol molecules then the production of this secondary metabolite decreases (45). So it was also concluded in many papers that whenever MS media is supplemented with tyrosol the grown cultures will show good accumulation of salidroside content in its parts (46).

In one of the papers it was reported that when the growth media is complimented with various known elicitors then different parts and tissues of the plant showed high variations in quality profiles (47). With over response of gene TyrDC it was noticed that the final collection of salidroside in the various parts of the plant, especially the roots was much increased to many folds (48).

This TyrDC gene is reported to be acting as substrate gene for tyrosol and thus for production of salidroside but with some limitations (49). UDP-glucosyltransferase is also another reported gene for production of this secondary metabolite, in the last stages (50). It transfers one molecule of glucose to the phenol molecule for its functioning (51) with help of UDP-glucose transferase (UDPG) working as the catalyst in the reaction (52).

- **Rosavin**

Rosavins belongs to the class of Cinnamoylglycosides and actually are the phenylpropanoids These were fir (53). Its function is to stimulate the motor activities which are unrestricted, against stress, and adaptogenic nature (54).

It is a product of phenomenon of phenylpropanoid absorption, which is derived from phenylalanine, which in turn is further an alternate for shikimic-chorismic disparaging pathway. The most studied gene for rosavin synthesis pathway is PAL gene and it does the destructive conversion of phenylalanine to form cinamic (55). The most common intermediates of this pathway Cinnamyl CoA ester which is formed from degradation of cinnamic with the help of enzyme 4CL. Another enzyme CCR reduces the above formedt CoA ester to a different product that is cinnamaldehyde. Other metabolite of this pathway is Rosin, the most effortless glycoside of roseroot is formed by one glucose trade (56). Rosin further gives rise to rosavin and rosarin (57).

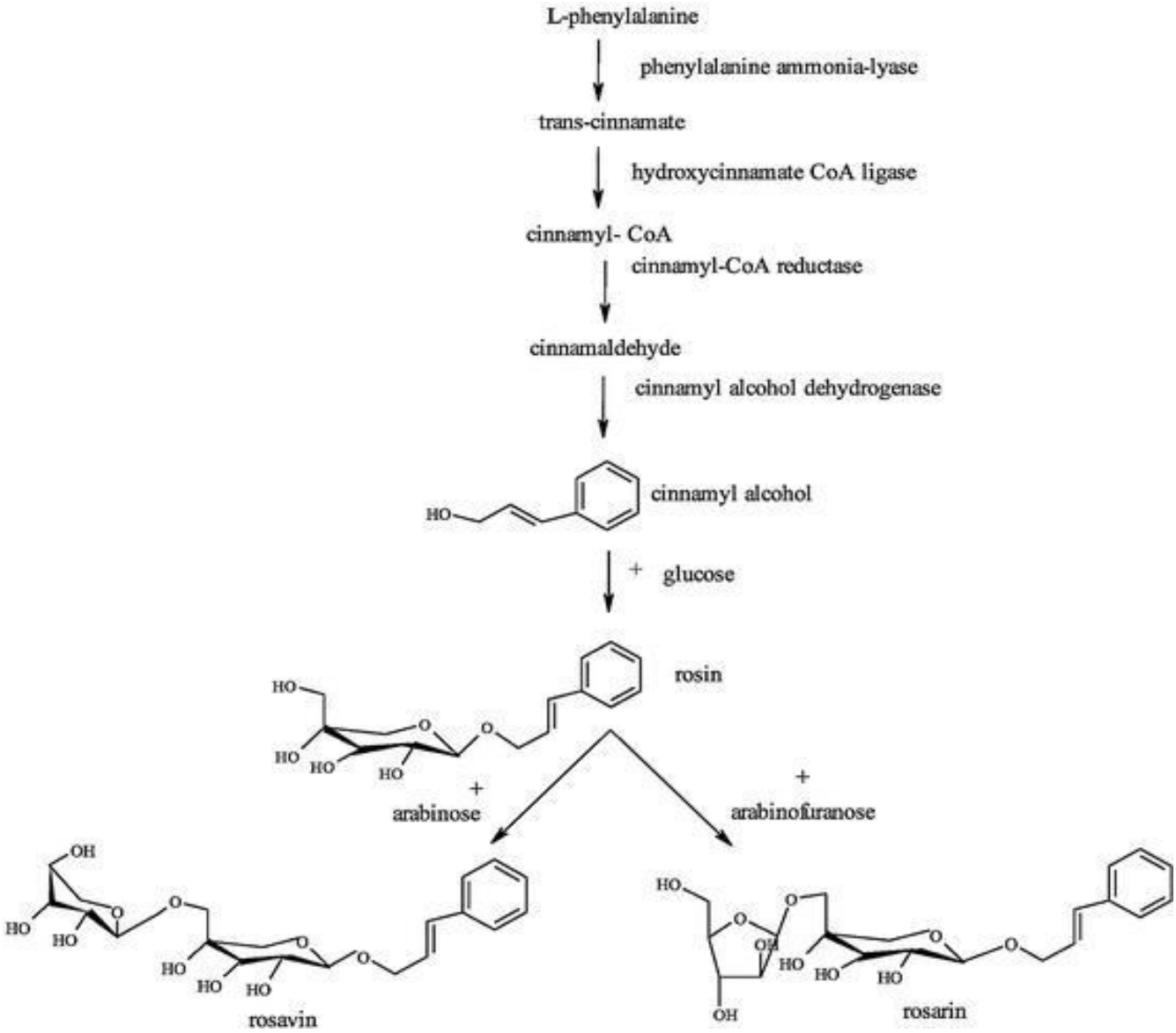


Fig 3. formulated pathway for rosavin (58).

CHAPTER NO- 3

MATERIALS

AND METHODS

Accumulating salidroside and rosavin by developing contrasting conditions for further use in transcriptome analysis

In callus and shoot cultures salidroside and rosavin being collected by optimizing media with various growth hormones combinations

Diverse MS media supplemented with different obsessions and mixes of Indole-3-butyric destructive (IBA), Kinetin (KN), 6-Benzylaminopurin (BAP), Gibberellic destructive (GA3) and Thidiazuron (TDZ) (below given table), sucrose 4% (w/v) to be prepared. pH conditions were set at five-seven with help of hydrochloric acid and sodium hydroxide in conclusion with some agar as gelling agent. Every type of combination was autoclaved properly and in vitro grown plant explants were taken comprising of shoot and callus. Prepared explants were sterilizedin uncontaminated conditions. Thus prepared culture were kept in culture rooms at 15 ± 2°C and 25 °C for around 16 h day/8 h night photoperiod.

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)

Key genes identification which are involved in Salidroside and Rosavin production

Isolation and verification of RNA

RNAqueous-Micro total RNA isolation kit (Life Technologies, AM1931) with modifications as follows:Elute RNA from beads by applying two hundred µL of lysis buffer on beads. Vortex on low speed for five to four times. Extract buffer from beads into a contemporary RNase-free tube. Add one hundred µL along with 100% alcohol then measuring system up and right down to combine resolution. Load sample onto columns and wash as delineated in kit protocol, then rinse with eight µL extraction buffer (supplied with the kit)—Repeat extraction double times.

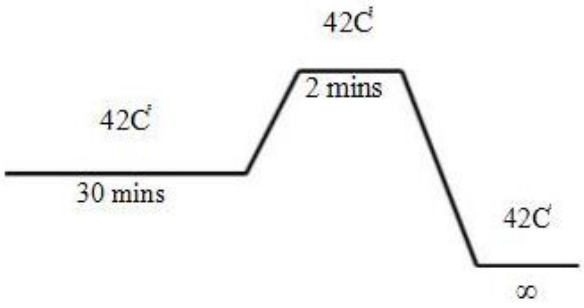
Synthesis of cDNA and verification on gel

Vortex solutions and centrifuge in brief previous to use. Organize the priming mix on ice in AN RNase-free reaction tube that is mentioned below. Combine softly by pipetting. Incubate samples at forty five °C for thirty min. If development of haphazard hexamers, incubate at ten min at twenty five °C followed by forty five °C for thirty min. Finish reaction by incubating at 85°C for five min, chill on ice. Store reaction at –20 °C for long run storage, or proceed to PCR directly.

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 – 94°C for 30 secs.
- Annealing – 58°C for 45 secs
- Extension – 72°C for 45 secs.
- Final Extension – 72°C 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 o r 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 o r C o r G o r T	a**N** y

Designing of specific primers for genes involved in synthesis of Rosavin

Coding groupings (Compact discs) of various species were recovered from NCBI database for Buddy, 4CL, CCR and computer aided design qualities. The arrangements recovered were adjusted through different grouping arrangement utilizing DNASTAR MegAlign and an agreement succession was gotten for every quality. The part of agreement grouping having least holes was utilized to plan degenerative preliminaries (Table 2) for every quality utilizing Preliminary 3 programming.

Amplification using PCR

PCR response blends of twelve and a half µl consisting Polymerase Chain Reaction support, dNTPs, forward and turn around primers of particular qualities, cDNA, Taq polymerase aand at last nuclease free water had to be added.

Further Gradient PCR had to be used at diverse temperatures profiles for about forty cycles to set the melting temperature for every thus designed primer at stated reaction setting. Subsequent to achievement of process, results were checked on 1.2 percent agarose gel to ensure that we got required results.

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

Amplicon extraction from gel and its purification

After upgrade, gatherings of needed ranges had to be separated from the gel under Ultra-Voilet illuminator and if we see any amplifications the sifted using gel extraction kit. Final products had to be run on 1.2% Agarose gel in 1X TAE pad for ensuring the desired results and further verifying it at 260 nano meter using Nano drop.

Sequence derivation

When Purging is done, 30 µl of cleansed items alongside 10 µl (10 µM) of groundworks of particular qualities were given to Xcelris Labs Restricted, Ahmedabad, Gujrat, India for performing Sanger sequencing method on prepared samples utilizing Huge Color Eliminator v3.1 pack.

Sample being prepared for Transcriptome investigations

Tissue samples included callus cultures grown at 15C and 25C and in-vitro grown shoots at the same temperature profiles, after quantification were used for Transcriptome generation. Qiagen RNeasy Plant Small scale Unit had to be used for unfastens ads to RNA samples using given protocol. Further sent to Eurofins Genomics India Pvt. Ltd.

Expression analysis

The cDNA concentrations were optimized and mixed with gene-specific forward and reverse primer pair. Following mixture was prepared in each optical tube:

- 12.5 µl SYBR Green Mix (2x)
- 0.2 µl cDNA
- 1 µl primer pair mix (5 pmol/µl each primer)
- 11.3 µl H2O

Following conditions were followed for PCR set up:

1. Initial Denaturation at 95ºC for 3 min
2. Denaturation at 95 ºC for 10 s
3. Annealing for30 s at Varying Temperatures (48ºC - 58ºC)

} 38 cycles

4. Extension at 72 ºC for 1min
5. Storage at 4ºC for infinite time

Dissociation curve was analyzed.

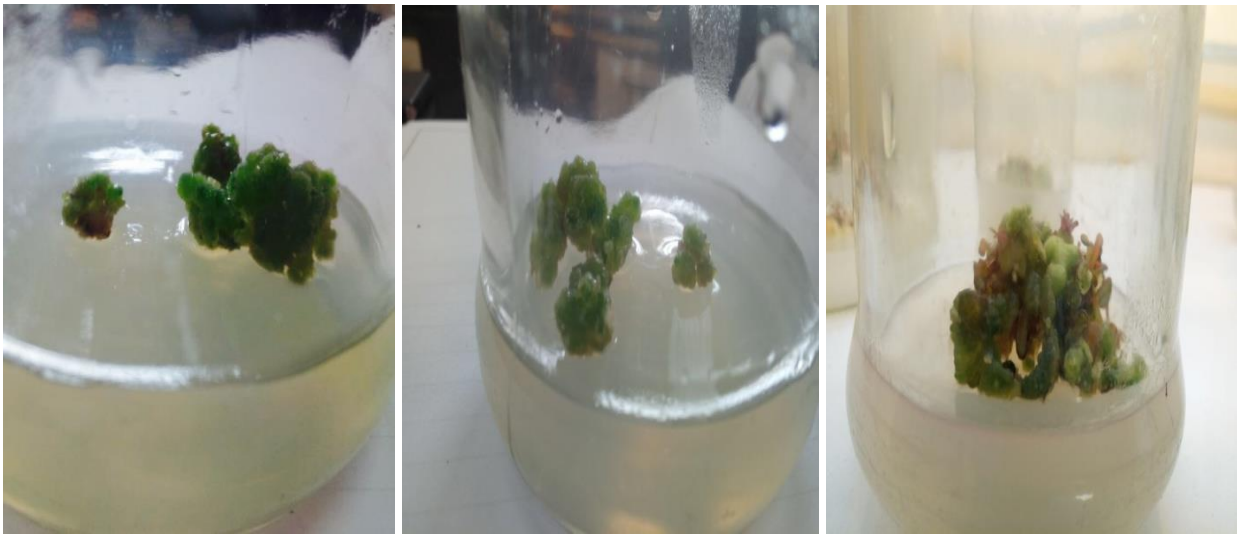
CHAPTER NO - 4

RESULTS AND DISCUSSION

Contrasting conditions are being developed for for accumulation of rosavin and salidroside for further processes

Prepared MS media being optimized along with combinations of growth hormones for production of rosavin and salidroside in in-vitro grown callus

among the 7 prepared mediacombinations, callus grew in only six combinations i.e. in. RI 2, 3, 4, 5, 6 and 7, all grown at 15 ± 2°C and 25°C. Combinations of BAP along with IBA in medium gave excellent results in callus with good amount of Salidroside. Rosavin traces were not observed in the samples tested.



a. **b.** **c.**

Figure 4. Callus growth from leaf used as explant. a.) leaf explant b.) Callus growth (in 15 days) c.)Sub-cultured callus (30days).

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,	Name of media	Days required For callus induction	Color of callus	Total Percent Calli (%)	Final 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**	$\mathbf{92\pm0.57^{f}}$	$\mathbf{2.401\pm0.05^{f}}$
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}

Various hormone combinations for optimizing the shoot and callus growth and initiation in MS media

Among the tried 7 blends, shoot initiation was started in three media blends which were RI 1, 3 and 7 at 15±2°C and 25°C. Combinations of BAP and KN was pragmatic to be the most excellent for the shoot increase with high secretion of Salidroside while MS medium supplied with IBA and KN was observed to be the less effective for the shoot augmentation with decreased secretion of Salidroside (Table 6). Rosavin traces were not at all found in the samples tested.

a. b. c.

Figure 5. 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±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	-----	-----	-----	-----

Key genes are identified responsible for rosavin and salidroside production

Isolation of RNA

RNA secluded from self initiated Shoots and Callus (green) cultures of our plant was checked on one percent agarose gel and two distinct amplifications of 18s had to be observed (Figure 10). The withdrew RNA was furthermore assessed using Nano drop and the data procured is depicted in Table 7.

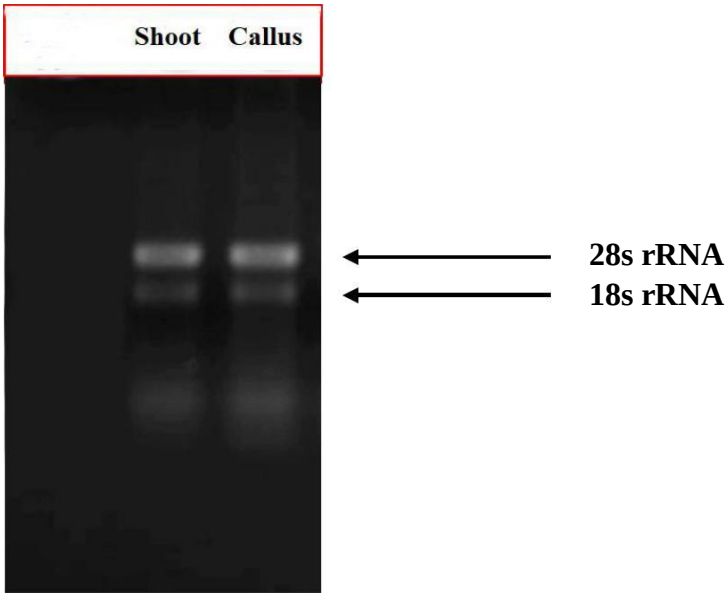


Figure 6. RNA isolated from in vitro grown Shoots and Callus of *R. imbricata*.

Table 7. 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	275	1.88	1.75
2.	in vitro Shoots	290	1.80	1.73

Construction of cDNA

Constructed cDNA was verified by amplifying through PCR utilizing specific primers for 26s rRNA. Amplified amplicons were verified by running on 1 % agarose gel and as results on gel brilliant amplified bands of 26s rRNA were seen at 500basepairs (Figure 7). Among these incorporated cDNA were likewise evaluated utilizing Nano drop and the information got was portrayed in Table given below.

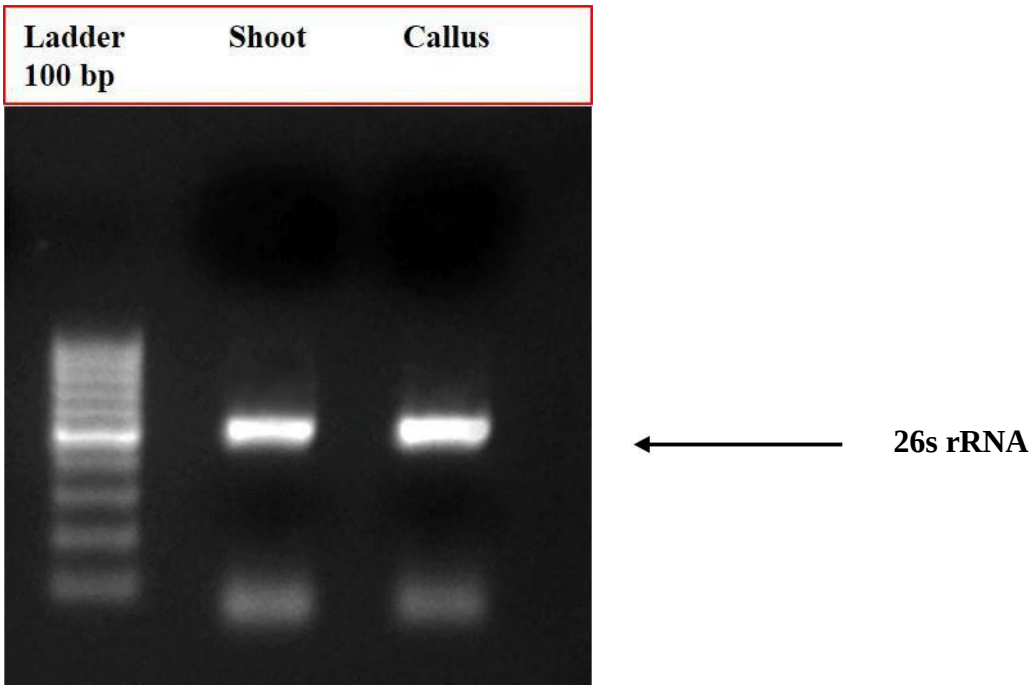


Fig 7. isolated cDNA from grown shoot and callus culture using specific 26s rRNA primers.

Table 8. 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	670	1.95	1.76
2.	in vitro Shoots	700	1.98	1.75

Specific primers designed for genes involved in salidroside synthesis

Sequences of specific primers for the key genes taking part in rosavin synthesis are mention in table:9 along with melting temperatures and amplicon sizes.

Table 9. 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	

Specific primers designed for genes involved in rosavin synthesis

Sequences of specific primers for the key genes taking part in rosavin synthesis are mention in table:10 along with melting temperatures and amplicon sizes.

Table 10. 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

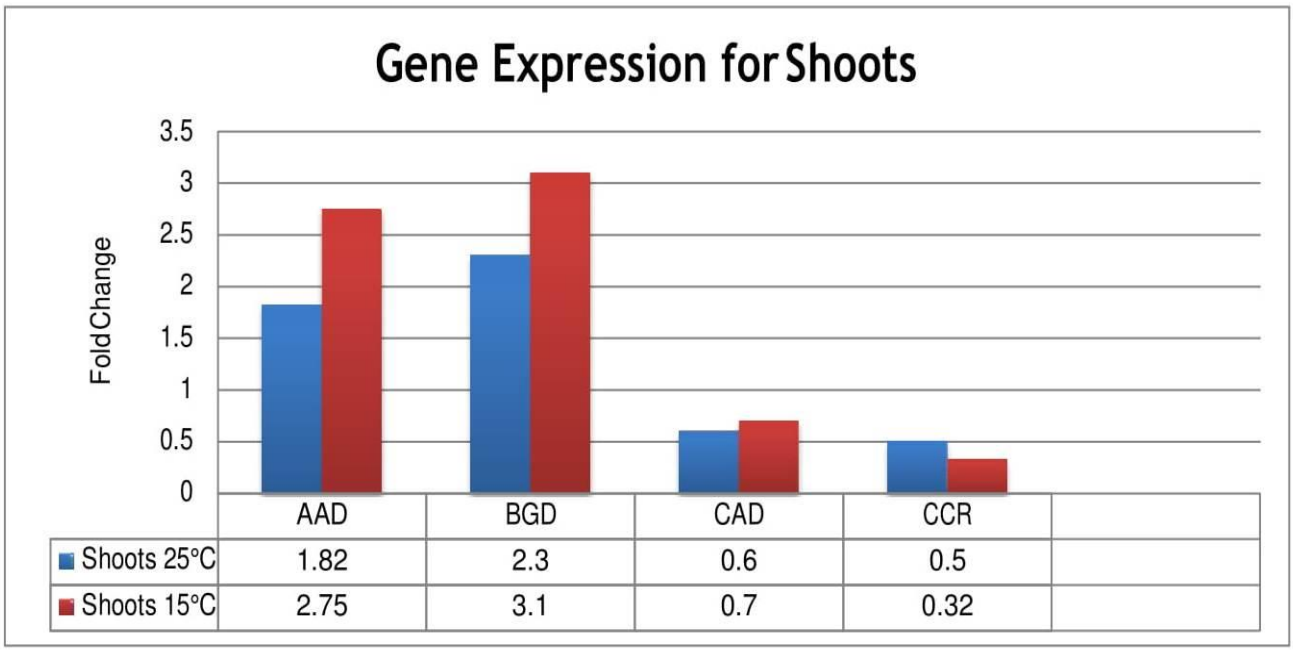
After finishing of PCR cycles, tests were keep running on 1.2% agarose gel (Figure 12) and Tm enhanced for every groundwork is recorded in Table 11.

Table 11. 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
SALIDROSIDE	TyrDC	50.5	536 bp
	UDPGT	57.0	501 bp
	β-GD	53.0	464 bp
	AAD	54.0	313 bp
ROSAVIN	PAL	59.0	413 bp
	4CL	59.0	436 bp
	CCR	58.0	399 bp
	CAD	58.0	372 bp

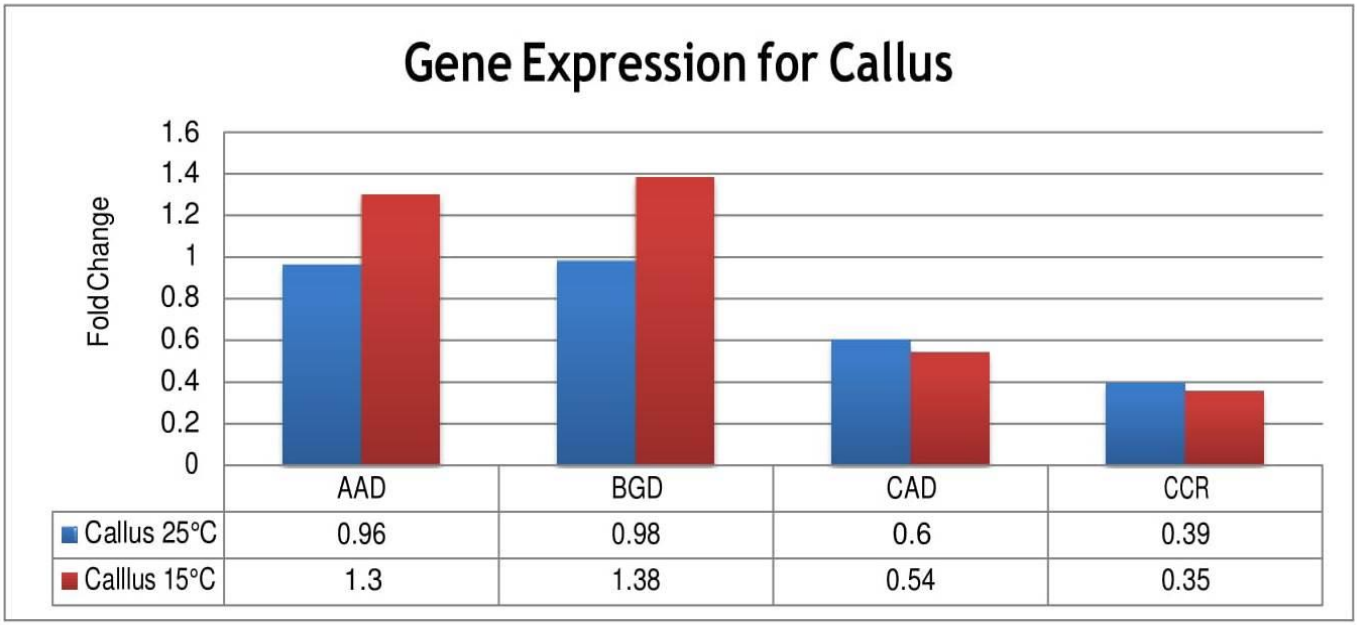
Gene expression analysis

Key genes AAD and BGD for Salidroside production were identified and their expression analysis was done and for Rosavin, expression analysis of CAD and CCR was done. Expression analysis was done on shoots and callus of *Rhodiola imbricata* grown at 15±2°C and 25±2°C. Fold change in gene expression was calculated. Shoots grown at 15±2°C showed 2.75, 3.1, 0.7 and 0.32 fold expression of AAD, BGD, CAD and CCR genes respectively. And shoots grown at 25±2°C showed 1.87, 2.3, 0.6, 0.5 fold expression of AAD, BGD, CAD and CCR genes respectively.



Graph1: Graphical representation of fold change in gene expressing for Salidroside (AAD; BGD) and Rosavin (CAD; CCR) in shoots grown at 15°C and 25°C.

Callus grown at 15±2°C showed 1.3, 1.38, 0.54 and 0.35 folds expression of AAD, BGD, CAD and CCR respectively. And Callus grown at 25±2°C showed 0.96, 0.98, 0.6, 0.39 fold expression of AAD, BGD, CAD and CCR genes respectively.



Graph1: Graphical representation of fold change in gene expressing for Salidroside (AAD; BGD) and Rosavin (CAD; CCR) in Callus grown at 15°C and 25°C.

DISCUSSION

My aim of the project was Optimizing contrasting environment for salidroside accumulation in different in-vitro grown tissues of *Rhodiola imbricate* and also expression analysis of primary genes responsible for biosynthesis of Salidroside and Rosavin in *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 does occur in rhizome of *R. imbricata* (Mishra et al., 2008).

Various surrounding situations were developed utilizing MS media and diverse development hormones blends for the buildup of both the metabolites and for transcriptome analysis. It was found that the MS medium having BAP along with IBA was finest for callus growth (59). Similarly, media combination of BAP along with KN gave best shoot growth. The successions got can be utilized for articulation concentrates to dissect the statement of these qualities under the specified differentiating conditions. We have likewise recognized AAD, BGD, TyrDC and UDPGT are the genes involved in the biosynthesis of secondary metabolites and imparting medicinal properties in *R.imbricata* and many other plants like alfalfa and Eucalyptus etc. plant species whereas in *R.imbricata* they gave 3 fold increase expressions of rosavin and salidroside in shoots when contrasted with callus. Shoot cultures at both temperature profiles showed higher level of metabolite accumulation as compared to callus.

Good growth and more accumulation of salidroside are noticed in shoots and callus whereas there was almost null accumulation of rosavin. Almost same growth and metabolite production was seen at 15ºC as well as 25ºC grown cultures. Expression of stated genes is not very significantly different from one other in callus tissues at 15ºC as well as 25ºC, whereas expression of these genes in shoot cultures as compared to callus are much higher in in-vitro grown shoot cultures. My report can be used by industries for production of these secondary metabolites for the medical requirements of the nation.

This plant is an imperiled restorative type which ensures its atomic investigation for the production of the required metabolites in different types of conditions and environment giving various medical benefits (60).

CHAPTER NO. - 5

CONCLUSION

R. imbricata is otherwise called golden root due to its abundant pharmacological exercises because of the nearness of auxiliary metabolites in it. We have additionally built up the differentiating condition utilizing MS media and different development hormones and distinctive temperatures to discover the media blend and temperature where the most extreme measure of metabolite gets aggregated. Result showed MS medium compromising combination of BAP and IBA resulted in maximum production of Salidroside. Samples were further prepared for transcriptome analysis and additional molecular learnings. Our study additionally found the various nature nd properties and pathways of this plant which are involved in the production of the major secondary metabolites that are salidroside and rosavin. The successions got can be utilized for articulation concentrates to dissect the statement of these qualities under the specified differentiating conditions.. Expression of stated genes is not very significantly different from one other in callus tissues at 15ºC as well as 25ºC, whereas expression of these genes in shoot cultures as compared to callus are much higher in in-vitro grown shoot cultures.

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