CHAPTER 4

COMPARATIVE STUDY OF NORMAL AND HAIRY ROOT CULTURES OF *GLYCYRRHIZA GLABRA* L.
4.1 INTRODUCTION

Separation of target bioactive compounds and their further identification through traditional chromatographic techniques is time consuming as the sample mixture consists of various untargeted compounds along with numerous impurities. To overcome this, a new mass spectroscopic technique DART-MS was introduced by Cody in 2005 for profiling of secondary metabolites (Cody et. al. 2005), this technique avoids a necessary step of sample preparation (Banerjee et. al. 2008a). DART-MS technique is widely used for detection of secondary metabolites but presently is being used for the first time in *G. glabra* hairy roots for recognition of secondary metabolites.

Various medicinal plants and their components have valuable pharmacological properties. Reports are available which showed antioxidant activity in many plants including *Ocimum sanctum, Allium sativum, Terminalia bellerica, Piper cubeba, Zingiber officinale, Camellia sinensis*, and several Chinese and Indian plants. The antioxidant activity is due to the presence of isoflavones, flavones, anthocyanin, flavonoids, coumarin, lignans, isocatechins and catechins (Aqil et. al. 2006). Literature showed the use of antioxidant-based drug formulations in prevention and treatment of complex diseases like stroke, atherosclerosis, Alzheimer’s disease, diabetes and cancer (Devasagayam et. al. 2004).

First time in 2008, a correlation was observed between the expression of individual rol genes and on the metabolism of reactive oxygen species ROS in plant cells (Bulgakov et. al. 2008). In *Rubia cordifolia* plant a study was performed to determine the connection between increased production of secondary metabolites and oxidative burst. Activation of cellular component is necessary for scavenging the ROS otherwise chain of free radical reaction was initiated. Free radicals damaged proteins, nucleic acids and lipids
like major biomolecules and finally lead towards disease conditions (Gülçın, 2006; Halliwell and Gutteridge, 1990). Antioxidant system present in living organism potentially cured the ROS and proved as beneficial in preventing loss caused by free radicals (Gülçın et al. 2004; Gülçın, 2010; Gülçın et. al. 2003; Tohma and Gülçın, 2010).

Proteins are directly related to their function due to this reason proteomics is the flattering branch to study in post-genomic era. The 2-D electrophoresis was recognized as a high resolution dominant technique for sorting proteins from complicated protein mixtures (Kim et. al. 2003b). Proteomics is used to compare proteomes under different conditions (Zhu et. al. 2013). Good sample quality is major prerequisite for proteomic approaches that’s why 2-D electrophoresis is difficult for analysing the plant samples. Presence of cell wall and phenolic compounds has been recognized as important barriers which reduces efficiency of protein separation on 2-D gels. An enriched quantity of oxidative enzymes and proteases in plant tissues create hurdle to extract stable protein mixtures. Besides this, low protein content in plant cells is an important constraint for effective extraction of proteins from plants (Ghosh and Xu, 2014). Use of TCA (trichloroacetic acid)—acetone precipitation along with phenol extraction method has been reported as a solution to certain extent to conquer all challenges regarding extraction (Isaacson et. al. 2006). In spite of some limitations, proteomics has been proved as well efficient for protein depiction of individuals, assessment of genetic variability, establishment of genetic distances, characterization of mutants and selection of proteins expressed during definite situation such as light, heat, cold, or hormone exposure (Kim et. al. 2003a; Marques et. al. 2001).

By analyzing the changes at transcript level during gene expression we cannot make any prediction about how changes occur at protein level. That is why independent
proteomics study is of high importance for analyzing the protein level differences. Proteins include enzymes, components of transcription and translation mechanism and much more (Kosova et al. 2011). Study of proteins is very potential approach for studying plant cells and plant secondary metabolism. Generally, the yield of secondary metabolites is less for its industrial applications, and chemical syntheses of these metabolites are costly and complicated too. Metabolic engineering is the most common strategy for enhancing the concentration of valuable metabolites. Hence, it is essential to identify the proteins involved in biosynthesis of targeted secondary metabolites along with other changes occurring at protein levels. Isolation and characterization of enzyme is very time consuming strategy. In contrast to this, the proteomic approach is much faster and more reliable. By using this technique identification of regulatory or transport proteins and enzymes would also be possible (Aghaei and Komatsu, 2013).

In the previous experiments, we have found high level of glycyrrhizin content in hairy root cultures of G. glabra. Keeping this in mind, we also examined expression of important genes of glycyrrhizin biosynthetic pathway and to observer protein level differences 2-D electrophoresis was performed. Higher level of differential expression of proteins was obtained in hairy roots comparison to normal root at SDS –PAGE level and also at 2- DE level. Moreover, the differentially expressed proteins were analyzed and identified by MALDI-TOF-MS.

4.2 MATERIAL AND METHODS

4.2.1 RNA isolation and qRT-PCR

Comparative expression profiling of important biosynthetic pathway genes of glycyrrhizin synthesis of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), farnesyl pyrophosphate synthase (FPPS), squalene synthase (SQS), b-amyrin synthase (bAS) and
CYP88D6 were examined through qRT-PCR in hairy roots and in control roots. Total RNA was extracted from fully grown normal and hairy roots using QiagenRNAeasy Plant Mini Kit (Qiagen, MD, USA) following the manufacturer’s Instructions. Total RNA was subjected to DNAse I (Ambion) treatment before use. Total RNA (5 μg) was used for synthesis of first strand of cDNA by using Revert Aid First Strand cDNA synthesis Kit (Fermentas, Life Sciences, ON, Canada). qRT-PCR was done in the Step One Plus Real-Time PCR System (Applied Biosystems 7500). Three independent biological replicates and for each biological replicate, three technical replicates were analyzed by qRT-PCR analysis. For normalizing the cDNA loading actin was used as reference gene. The relative gene expression was determined through comparative CT method.

4.2.2 Protein extraction for enzymatic activities

Normal root and hairy root were crushed in pre chilled pestle mortar in extraction buffer. Extraction buffer constitute 10mM potassium phosphate buffer (pH 7.8), with 1%PVP (w/v), 0.5% triton X-100(v/v) and 0.1mM EDTA. Homogenate was centrifuged at10, 000 rpm for 30min at 4 ºC, supernatant was collected and stored at -80 ºC for later analysis. The total protein of normal and hairy roots was determined by Bradford method. Bovine serum albumin (BSA, Sigma Aldrich, USA) was used as standard.

4.2.3 Antioxidant activity

Superoxide dismutase(SOD,EC1.15.1.1) activity was determined using Bayer and Fridovitch protocol (Beyer and Fridovich, 1987). The reaction mixture was consisting 100mM potassium phosphate buffer (pH 7.8), 10 mM-methionine, 0.025%(v/v)Triton X-100 and 0.57mM nitro blue tetrazolium chloride (NBT). 2mL from the solution was transferred into glasstubes, 20 μl of protein and 4.4% (w/v) riboflavin were also added to
the tube just before taking OD. Volume was made up by sterile water. After taking initial OD, tubes were illuminated for 7 min in light. In both cases OD was measured at 560 nm on PerkinElmer, Lambda 35, UV/VIS spectrophotometer.

Ascorbate peroxidase (APX, EC1.11.1.11) activity was measured according to Nakano and Asada (Nakano and Asada, 1981). For the assessment of ascorbate activity reaction mixture was prepared, and it consists of 50 mM potassium phosphate buffer, 5 mM ascorbate, 1 mM EDTA and 100 µL of the sample. 1 mM hydrogen peroxide was added lastly. OD was recorded by reading the absorbance at 290 nm continuously for 3 min.

Guaiacol peroxidase (GPX, EC1.11.1.7) activity was measured according to Zheng and Van Huystee protocol (Zheng and Van Huystee, 1992). The reaction mixture was made of 10 mM sodium phosphate buffer (pH 5.8), 1% guaiacol (v/v) and 100 mM H₂O₂. Absorbance was recorded at 470 nm, kinetics was measured every 10 second for 1 min.

Catalase (CAT, 1.11.1.6) activity was analyzed according to Aebi protocol (Aebi 1984). For catalase test, 50 mM sodium phosphate buffer (pH 7.0), 10 µl enzyme extract and 2 mM H₂O₂ was used in reaction mixture. The decrease in H₂O₂ was followed by a decline in absorbance at 240 nm.

**4.2.4 DART mass spectrometry**

The mass spectrometer used was a JMS-100 TLC (AccuTof) atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) and it is fitted with a DART ion source. The mass spectrometer contain resolving power of 6000 (full-width at half-maximum) and operated in positive-ion mode. For minimal fragmentation orifice 1 potential was set to 28 V. The ring lens and orifice 2 potentials were set to 13 and 5 V, respectively. Temperature of orifice 1 was set at 100 ºC. The RF ion guide potential was
300 V. For the operation of DART ion source, flow rate of helium gas was maintained at approximately 4.0 L min\(^{-1}\). The gas heater temperature was set at 300 °C. The electrode potential of the DART source was set to 3000 V, 100 V and 250 V for discharge needle electrode, electrode 1 and the grid, respectively. Fresh sample of hairy roots were situated in the gap between the DART source and mass spectrometer for analysis. Data acquisition was from m/z 10 to 1050. To ensure the reproducibility of the analysis fifteen repeats for each sample were performed. Exact mass calibration was achieved by including a mass spectrum of neat polyethylene (PEG) glycol (1:1 mixture PEG 200 and PEG 600) in the data file.

For analyzing the secondary metabolites the intact hairy roots and normal roots were held in front of DART ion source, instantly mass spectrum was obtained. The peaks at m/z corresponded to the [M+ H]\(^+\) (molecular formula) of the compounds. Compounds were identified according to Dr. Duke's Phytochemical and Ethnobotanical Databases.

![Figure 4.1: DART Spectrum of G. glabra. (a) Normal roots; (b) Hairy roots](image)

(a) (b)
4.2.5 Proteomic Analysis for 2-D electrophoresis

4.2.5.1 Protein extraction

Protein was extraction from roots of *G. glabra* according to Damerval protocol with little modification (Damerval et. al. 1986). Samples were crushed in liquid N\textsubscript{2} with the aid of mortar and pestle. The obtained powder was extracted with Tris-HCl (50 mM, pH 8.0), EDTA (25 mM), thiourea (500 mM) and β-mercaptoethanol (0.5%). The extract was blended with cold TCA (10%) with β-mercaptoethenol (0.07%) and left overnight at -20 °C. Centrifugation was performed at 4500 rpm for 10 min and the pellet was washed three times with acetone and BME (0.07%). The pellet was then vacuum dried and solubilized in Tris-HCl (0.1 M, pH 8.0), EDTA (50 mM) and BME (2%). Next step was performed with Tris buffered phenol (2.5 mL) and mixture was centrifuged at 4500 rpm for 10 min. After centrifugation, lower phenol phase was collected through Pasteur pipette. To this 10 mL, ammonium acetate (0.1 M) prepared in methanol was added and again left overnight at -20°C. Ten min centrifugation was performed at 4500 rpm and pellet was dissolved in ammonium acetate (0.1 M) and BME (1%). It was centrifuged at 6000 rpm for 10 min and two times washed with cold acetone. Dried pellet was resuspended in a solubilization buffer consisting of urea (7 M), Thiourea (2 M), CHAPS (2%), DTT (20 mM), 0.5% v/v immobilized pH gradients buffers. Quantification of proteins was done through the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as the standard.

4.2.5.2 2-D Gel electrophoresis

Two-dimensional gel electrophoresis (2-DGE) was performed as described in (Lehesranta et. al. 2005) with little modifications. Protein samples (200 µg) were rehydrated overnight on immobilized pH gradient (IPG) strips (11 cm, pH 3-10, linear) (Bio Rad )with 200 µL of rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 20 mM
DTT, 0.5% v/v immobilized pH gradients buffers) in a reswelling tray (Amersham Biosciences, Uppsala, Sweden) at RT. Isoelectric focusing (IEF) was conducted at 20 °C with an Ettan IPGphore-3 (GE Healthcare) and a Dry Strip kit (Amersham Biosciences, Uppsala, Sweden). The operated condition was as follows: 250V for 1 h, 500V for 1 h, 1500V for 2 h, and 4000V for 2 h and 6000V for 2 h for a total of 21.2 kVh. The focused strips were equilibrated twice in 5 mL of equilibration solution only for 10 min (I and II) (6M urea, 30% w/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), and 50 mM Tris-HCl buffer, pH 8.8). The equilibration solution I consist of 1% w/v DTT and equilibration solution II contains 2.5% w/v iodoacetamide instead of DTT. Second dimensions were carried out on polyacrylamide slab gels using the discontinuous buffer system. The separating gel of 12.5% polyacrylamide with an acrylamide: Bis ratio of 29:1. Standard Tris-Glycine running buffer in a Miniprotein III dual slab cell (Bio-Rad) at a constant 70V and 120V slab gel using a Power PAC300 (Bio-Rad) was used for electrophoresis. Cumassie brilliant blue G (0.5%) gels were used to stain gel and 10% glacial acetic acid in 50% methanol was used to destain the gel. Images were attained with document scanner; image analysis was performed using Image Quant TL 7.0 software (GE Health care). Analysis was performed through Image Master 2D Platinum 7.0 software (Amersham Bioscience). Relative volume (% volume) was used to quantify and compare the spots. Relative volume considers the ratio of detected spot pixel density to the sum of all analyzed spot pixel density. Hence, this procedure permitted to normalize experimental variations due to protein loading and staining. Experiments were performed in triplicate i.e. three gels of each sample were used in data analysis. Protein expression patterns were determined according to up-regulated, % volume increased at least one and half-fold;
down-regulated, % volume decreased at least one and half-fold; unchanged, % volume varied within one and half-fold.

4.2.5.3 Protein Identification

Protein spots were excised from the gels for tryptic digestion. Sample preparation was carried out according to (Koistinen et. al. 2002). Firstly gel particles were destained and dehydrated by three times washing with ABC (ammonium bicarbonate 25 mM) with 50% acetonitrile. Destained particles were dried in a vacuum centrifuge concentrator and rehydrated in equal volumes of 0.1 mg/mL trypsin and ABC (50 mM). Gel particles were immersed in ammonium bicarbonate (25 mM) and samples were digested overnight at 37°C. Peptides were extracted twice with 50% ACN/1% TFA, gel particles were rehydrated with water, and this procedure was repeated twice. The recovered peptides were concentrated to a final volume of 20 mL.

4.2.5.4 MS and MS/MS

For all MALDI-MS and MS/MS applications, A 4800 Proteomics Analyzer (Applied Biosystems) with TOF/TOF optics was used. Final samples were prepared by adding 0.5 mL of sample with 0.6 ul of matrix solution (5 mg/mL a-Cyano-4-hydroxycinnamic acid in 50% ACN with 0.1% TFA) and spotted on stainless steel 384 well target plate. Before insertion in the mass spectrometer for mass analysis, samples were allowed to air dry at RT. The mass spectrometer is externally calibrated with standards. These standards are mixture of angiotensin I, Glu-fibrino-peptide B, ACTH (1-17), and ACTH (18-39). For MS/MS experiments, the instrument was externally calibrated with fragment of Glufibrino-peptide B. The monoisotopic peptide masses obtained from MALDI-TOF/TOF were analyzed by the 4000 Series Explorer software version 3.5 (ABI). On the basis of mass signals, protein identification was performed with the Mascot
software (http://www.matrixscience.com) to search proteins against Swiss Prot, NCBI
and MSDB databases. The following parameters were used for database searches:
monoisotopic mass accuracy, <100 ppm; missed cleavages, 1; carbamidomethylation of
cysteine as fixed modification and oxidation of methionine, N-terminal pyroglutamylata
tion (peptide) and N-terminal acetylation (protein) as variable modifications.

4.3 RESULTS

4.3.1 Transcriptional response of MVA pathway genes involved in glycyrrhizin
biosynthesis

Glycyrrhizin synthesis occurred through mevalonate pathway (MVA). Elevated
level of glycyrrhizin in hairy roots (examined through HPLC) was further investigated by
the transcript level of the intermediate glycyrrhizin biosynthetic pathway genes (HMGR,
FPPS, SQS, bAS and CYP88D6) through qRT-PCR. Transcript profiling of these genes
showed enhanced levels in hairy roots in comparison to normal roots. Expression level of
HMGR was 5.6 fold higher, for FPPS 2.4 fold, for SQS 1.5 fold, for bAS 1.6 fold and for
CYP88D6 4.9 fold increase was observed (Figure 4.3). HMGR is the rate limiting enzyme
of MVA pathway and CYP88D6 is the last step enzyme of glycyrrhizin synthesis. Results
obtained through qRT-PCR analysis, clearly demonstrates the effectiveness of insertion of
Ri-plasmid at gene level.
Figure 4.2: Systematic representation of glycyrrhizin biosynthesis pathway.
**Figure 4.3**: Quantitative expression of HMGR, FPPS, SQS, hAS and CYP88D6 genes in hairy root and normal root.
### Table 4.1 Primers used in the study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>ActinF</td>
<td>GATGCCTATGTGGGTGATGAA</td>
</tr>
<tr>
<td>ActinR</td>
<td>GATCTTTTCCATGTCATCCAG</td>
</tr>
<tr>
<td>HMGRF</td>
<td>GGTGCTCTGGGTGGTTCA</td>
</tr>
<tr>
<td>HMGRR</td>
<td>CTCTCCACATTCTGAGCAGGATC</td>
</tr>
<tr>
<td>FPPSF</td>
<td>TGGACTACAATGTGCCTGGAG</td>
</tr>
<tr>
<td>FPPSR</td>
<td>AGCCATTCACATACACCAACCAAG</td>
</tr>
<tr>
<td>SQSF</td>
<td>AGTTCCCTCCCGGTGTTCTT</td>
</tr>
<tr>
<td>SQSR</td>
<td>GCTCCCAAACTCCCCATAGT</td>
</tr>
<tr>
<td>bASF</td>
<td>CACTTCTGACTCGTTGGCCT</td>
</tr>
<tr>
<td>bASR</td>
<td>CTTCACCACCAACAGCAAGC</td>
</tr>
<tr>
<td>CYPF</td>
<td>GCTAAGGAAGAGGAGGAAAGAAAT</td>
</tr>
<tr>
<td>CYPR</td>
<td>CTACTTCTGTCCAAAAAGGAA</td>
</tr>
</tbody>
</table>

### 4.3.2 Enhancement in SOD activity after elicitation

Normal root and hairy root cultures were investigated for assessment of their enzymatic and non-enzymatic antioxidant activities. In hairy roots, elevated level of APX (1.5 fold), CAT (2.1 fold), GPX (1.3 fold) and SOD (2.6 fold) were found in comparison to normal roots (Figure 4.4). As SOD is the main antioxidant enzyme and highest increase was obtained in its activity in hairy roots in comparison to other antioxidant activities. We have also examined SOD activities in control and stress conditions in both normal roots and hairy roots. Both kinds of roots were subjected to drought, cellulase and mannan stress. We have determined SOD activity at optimized concentration and duration of stress.
Optimized conditions were chosen on the basis of highest increase in glycyrrhizin concentration in HPLC analysis. PEG at 1% concentration after 24 h enhanced the SOD activity to 3.6 fold and 2.6 fold in normal and hairy roots, respectively (Figure 4.5a). Enhancement in SOD level was also obtained after 200 µg/mL cellulase concentration after 7 d post stress, in normal root enhancement was 8.4 fold and in hairy roots it was 5.2 fold (Figure 4.5b). Mannan at 10 mg/L concentration enhanced the SOD activity up to 1.6 fold in both normal and hairy roots after 10 d of treatment (Figure 4.5c).

![Antioxidant enzyme activity in normal roots and hairy roots. (a) GPX; (b) SOD; (c) APX; (d) CAT.](image)

*Figure 4.4: Antioxidant enzyme activity in normal roots and hairy roots. (a) GPX; (b) SOD; (c) APX; (d) CAT.*
4.3.3 Comparative DART-MS analysis

For identification and differentiation of bioactive compounds, DART-MS analysis was performed in hairy roots and normal roots of *G. glabra*. Twenty three peaks were obtained in both root spectra among them thirteen compounds were identified and mentioned in Table 4.2. Spectra of both roots, does not provide any significant difference.

Table 4.2  DART - MS Mass measurement of phytochemicals in hairy roots of *G. glabra*.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the compound</th>
<th>Molecular weight</th>
<th>[M+1]⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O acetyl salicylic acid</td>
<td>180.1574</td>
<td>180.1844</td>
</tr>
<tr>
<td>2</td>
<td>6-Methyl-5-hepten-2-one</td>
<td>126.1961</td>
<td>127.1087</td>
</tr>
<tr>
<td>3</td>
<td>Isoflavone</td>
<td>222.2387</td>
<td>223.2513</td>
</tr>
<tr>
<td>4</td>
<td>Butylphthalate</td>
<td>278.34</td>
<td>279.3184</td>
</tr>
<tr>
<td>5</td>
<td>Dodecane</td>
<td>170.3348</td>
<td>171.2220</td>
</tr>
<tr>
<td>6</td>
<td>Furfurylformate</td>
<td>126.1100</td>
<td>127.1087</td>
</tr>
<tr>
<td>7</td>
<td>Linalooloxide</td>
<td>170.25</td>
<td>171.2220</td>
</tr>
<tr>
<td>8</td>
<td>Maltol</td>
<td>126.11</td>
<td>127.1087</td>
</tr>
<tr>
<td>9</td>
<td>Tetradecane</td>
<td>198.388</td>
<td>199.2114</td>
</tr>
<tr>
<td>10</td>
<td>Octanoic acid</td>
<td>144.2114</td>
<td>144.1448</td>
</tr>
<tr>
<td>11</td>
<td>Umbellifeone</td>
<td>162.1421</td>
<td>163.1485</td>
</tr>
<tr>
<td>12</td>
<td>Dodecanoic acid</td>
<td>200.3177</td>
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</tr>
<tr>
<td>13</td>
<td>Linolenic acid ethyl ester</td>
<td>306.48</td>
<td>306.2999</td>
</tr>
</tbody>
</table>
4.3.4 Proteomics analysis

In *G. glabra* roots, more than 340 protein spots were reproducibly detected on brilliant blue G (comassie) stained gels, out of which 162 spots were matched between control and line gels. Out of 84 differentially expressed (P < 0.05) proteins spots, 64 spots were identified by MALDI-TOF/TOF. Twenty five protein spots (39%) were up-regulated while 39 spots (61%) were down regulated (Figure 4.8). These protein spots were categorised in eight different functional categories viz., carbon metabolism (20%), defense (20%), protein synthesis assembly degradation (17%), flavonoid biosynthesis (11%), energy Metabolism (10%), Amino Acid Metabolism (8%), Photosynthesis (3%), Transportation (2%) and proteins of unknown functions (9%) (Figure 4.9).

Most of the proteins involved in carbon metabolism were up-regulated clearly showing increased carbon allocation to hairy root resulting in increased biomass. Six proteins were involved in energy metabolism out which four were up-regulated and two were down regulated. Protein synthesis assembly and degradation (PSAD) related proteins (11 nos) were identified and most of them were down regulated. Proteins involved in defense have shown mixed expression pattern i.e. some of them were up-regulated and some down regulated. Some other functional categories like flavonoid biosynthesis and amino acid metabolism have also played important role to giving strength to hairy root over normal one (details of all identified proteins is mentioned in appendix-1).

Up-regulated proteins: - Eukaryotic initiation factor 4A-11 (spot ID-2), lactoylglutathionelyase [(spot ID-55) and Heat shock 70 kDa protein 4 (spot ID-185) were found highly up-regulated in hairy root cultures. These proteins involved in protein synthesis assembly and degradation. 5-methyltetrahydropteroylglutamate-homocysteine methyltransferase 2-like (spot ID-4) and glutamine synthetase PR-2 (spot ID-107) were
found up-regulated and involved in amino acid metabolism. Some defence related proteins as thaumatin-like protein PR-5b (spot ID-18), cytosolic ascorbate peroxidase (spot ID-59), chitotriosidase-1-like (spot ID-143), chitotriosidase-1-like (spot ID-128) and chitinase (spot ID-61) were found to be significantly up-regulated in hairy roots. Some proteins involved in flavonoid biosyntheses such as chalcone-flavononeisomerase 2 (spot ID-34) and isoflavonereductase-like protein (spot ID-53) were also up-regulated. Major proteins involved in energy metabolism were found significantly up-regulated in hairy root cultures of G. glabra in contrast to normal root. These are transaldolase (spot ID-119), NADPH-specific isocitrate dehydrogenase partial (spot ID-127), ATP synthase subunit beta, mitochondrial (spot ID-153), V-type proton ATPase subunit B2 (spot ID-149). Proteins involved in carbon metabolism were also upregulated like, 2,3-bisphosphoglycerate-independent phosphoglyceratemutase-like (spot ID-176), 2,3-bisphosphoglycerate-independent phosphoglyceratemutase-like (spot ID-175), alcohol dehydrogenase isoform X1 (spot ID -115), formate dehydrogenase (spot ID-112), malate dehydrogenase, mitochondrial (spot ID-99), malate dehydrogenase, cytoplasmic-like (spot ID-96), probable fructose-bisphosphatealdolase 3, chloroplastic (spot ID-77), probable fructose-bisphosphatealdolase 3, chloroplastic(spot ID-67) and triosephosphateisomerase, cytosolic (spot ID-43).

Down- regulated proteins:- Proteins involved in carbon metabolism like aldehyde dehydrogenase family 2 member C4-like, aldehyde dehydrogenase family 2 member B7 (mitochondrial-like), succinate dehydrogenase [ubiquinone] flavoprotein subunit (mitochondrial), NADP-dependent malic enzyme-like protein were down regulated. Some proteins like Purple acid phosphatase 27-like precursor, Glycoside hydrolase (family 17) were down regulated and were related to energy metabolism. Proteins involved in protein
synthesis assembly and degradation (PSAD) like drought-induced protein RPR-10, 20S proteasome subunit PBA1, Ferritin-3 (chloroplastic), cysteine proteinase, elongation factor Tu (mitochondrial), calreticulin 2, protein disulfide isomerase, endoplasmic reticulum HSC70-cognate binding protein precursor were significantly down regulated. Proteins involved in flavonoid biosynthesis like Isoflavonereductase and chalcone-flavononeisomerase 2 were down regulated. In defence, Minor allergen Alt a 7-like, 2-Cys peroxiredoxin BAS1 (chloroplastic), cytosolic ascorbate peroxidase, endochitinase A2-like precursor, chitinase, peroxidase 1 precursor and monodehydroascorbatereductase (chloroplastic) were found to be down regulated. Proteins involved in amino acid metabolism like Serine hydroxymethyltransferase 4, S-adenosylmethionine synthase, cysteine synthase-like were down regulated. Besides these some unknown functions proteins were also down regulated.

**Figure 4.6:** Protein profiling of normal Vs hairy roots through SDS-PAGE.
Figure 4.7: Representation of root protein spots of *G. glabra* stained through commassie blue dye in 2-D electrophoresis gel. (a) Normal root; (b) Hairy roots.
Figure 4.8: Pie diagram showing expression pattern of proteins in normal and hairy roots (induced after insertion of Ri-plasmid from *A. rhizogenes*). (a) Number of up or down regulated proteins; (b) Protein expression in percent.

Figure 4.9: Functional categorization of root proteins of *G. glabra* obtained from two-dimensional gel electrophoresis and identified though Maldi-TOF/TOF.
4.4 DISCUSSION

*Rol* genes induced changes in glycyrrhizin biosynthesis were observed through metabolite and transcript profiling. Fivefold enhancement was observed in glycyrrhizin content over normal root through HPLC analysis. Gene expression analysis of important MVA pathway enzymes involved in glycyrrhizin biosynthesis was performed through qRT-PCR. Expression of *HMGR, SQS, FPPS, bAS* and *CYP88D6* genes was found higher in hairy root in contrast to normal roots. Elevated expression level of all important glycyrrhizin pathway genes supported high accumulation of glycyrrhizin in hairy roots. Transcriptomic and metabolomic studies proved the strong connection between production of secondary metabolite and expression of their corresponding biosynthetic genes (Matsuda et al. 2010).

Over expression of *HMGR, FPPS* and *SQS* in the mevalonate pathway leads towards enhancement in triterpenoid accumulation (Kim et al. 2010; Munoz-Bertomeu et al. 2007; Seo et al. 2005). *SQS* and *bAS*, two genes, design the backbone of triterpene (Hayashi et al. 1999; Hayashi et al. 2001). Biosynthesis of glycyrrhizin (triterpenoid saponins) started from initial cyclization of 2, 3-oxidosqualene through *bAS*. *CYP88D6* converted beta amyrin to 11-oxo-b-amyrin through two oxidation steps. 11-oxo-b-amyrin is an expected intermediate between beta amyrin and glycyrrhizin.*CYP88D6*, Cytochrome P450 enzyme is exclusively present in Fabaceae family (Seki et al. 2008; Seki et al. 2011).

Higher antioxidant activity was found in hairy roots in comparison to normal roots. Mode of induction and their further cultivation was one of the possible reasons behind this higher level of antioxidant activity. Presence of TL region with *rol* genes are responsible for genome wide differences that exist in these transformed roots in contrast to
normal roots (Georgiev et al. 2010). NADPH oxidase system was induced for activation of ROS after integration of rolB gene. To protect the cells from massive damage of ROS activities, defence system of cells was activated in the form of elevated level of enzymatic and non enzymatic antioxidant system (Shkryl et al. 2010). SOD activity was also analyzed in normal and hairy roots after elicitation experiments. Both kinds of roots were subjected to drought, cellulase and mannan stress, enhancement in SOD activity was observed after elicitation but fold increase was higher in normal roots as compared to hairy roots after drought and cellulase stress. Reports are also available which showed infection of A. rhizogenes and presence of rolB and rolC genes makes plant roots liberal towards abiotic stress and activates the plant defence system via inducing secondary metabolite and PR protein synthesis (Bulgakov et al. 2013). Drought stress is certainly coupled with oxidative stress; enhanced accumulation of ROS occurred in chloroplasts, peroxisomes and mitochondria after drought stress. Efficiency of plants to conquer with oxidative stress fairly depends on the stimulation of SOD activity. To some extent, SOD activity is substrate dependent, increase in ROS level promoted gene encoding SOD and SOD act like a scavenger in degrading ROS level (Abedi and Pakniyat, 2010). Previous reports are in favour with our observations, as they already reported enhancement in SOD activity in reaction to drought stress in sunflower (Gunes et al. 2008), in wheat (Bakalova et al. 2004) and also in G. uralensis (Pan et al. 2006).

Mass spectrometry proved as important technique in biotechnology for identification of compounds. DART–MS, an ionization technique coupled with a time-of-flight mass spectrometer has quite higher sensitivity. DART is very useful and widely used technique in identification of organic compounds in drugs, in body fluids, in food and it is also useful in forensic sciences. This technique was used for the identification of reserpine
and vomilenine from hairy root cultures of *R. serpentina* (Madhusudanan et. al. 2008). In hairy root cultures of *Atropa acuminata* atropine and scopolamine alkaloids were identified through DART-MS techniques (Banerjee et. al. 2008a). Beside these two reports, there is no report available regarding DART-MS profiling in hairy roots. In our study thirteen compounds were identified from hairy root culture of *G. glabra*.

Proteomics study was performed between normal and hairy roots. The 2-D pattern of the normal roots of *G. glabra* significantly different from that of the cultured hairy roots, suggesting that high level of protein differences were found in both types of roots. Among 84 differently expressed spots 64 spots were identified through MALDI-TOF/TOF analysis. These identified spots were functionally categorized (Table 4.3). Hairy roots are induced due to insertion of Ri plasmid in plant genome from leaves and further their proliferation was performed in hormone free medium. On the other hand normal roots induced from in *vitro* shoots in the presence of IAA (0.05mg/L).

Although before protein extraction culture conditions were kept identical for normal (wild type roots) and hairy roots but their origin and proliferating conditions were different and these altered conditions may be one of the reasons for proteomic level differences. Similar kind of differences was also observed by Kim et. al. (2003b) between cultured hairy roots and main roots of ginseng due to different environment and culture conditions.

Thaumatin-like protein are group of pathogen related proteins (PR-Proteins), these proteins are expressed in plants in response to microbial infection and other stresses (Datta et. al. 1999). Higher expression of this protein in hairy roots was quite obvious as these roots are induced due to microbial infection. Evidence are also available which showed that *rol* genes activate the transcription of defense related genes (Bulgakov et. al. 2011)
Some other defense related proteins such as cytosolic ascorbateperoxidise, chitotriosidase-1-like and chitinase were also found up-regulated in hairy roots. We also obtained higher level of antioxidant enzyme in the form of elevated level of SOD, GPX, APX and CAT in biochemical assays. Many researchers have reported that abiotic stress like conditions such as wounding etc. responsible for enhancing the level of one or more antioxidant enzymes (Grantz et. al. 1995, Van Camp et. al. 1996).

In *Rubia cordifolia* calli transformed with *A. rhizogenes* A4 strain and decrease in ROS level was obtained. The repression of ROS in transform cells was associated with increase expression of many genes encoding ROS-detoxifying enzymes (Shkryl et. al. 2010).

The advantages associated with hairy root cultures are their fast growth without additional hormone supply, their genetic and biochemical stability and also their potential to synthesize bioactive compound at levels comparable to or even higher than those found in normal roots of the plants (Skala et. al. 2015). In our study two protein spots chalcone-flavononeisomerase 2 and Isoflavonereductase-like proteins involved in flavonoid biosynthesis were found up-regulated in hairy roots. Although some proteins like isoflavone reductase and chalcone-flavonone isomerase 2 with different subunits were found to be down-regulated in hairy roots.

Studies show the rate of plant growth depends on the rate of carbon metabolism and also on the developmental programs that determine and ensure the effective conversion of carbon into biomass (Sulpice et. al. 2010). On the basis of this fact we can correlate the high biomass production and significant up-regulation of enzymes of carbon metabolism. As in our study we identified 9 spots involved in carbon metabolism significantly up-regulated in hairy root cultures. Expression of *rolB* and *rolC* genes plays principle role in
the induction of hairy roots. High expression of the rolB gene significantly enhanced the biosynthesis of bioactive compounds (Shkryl et al. 2008), conversely over expression of rolB gene inhibited cell growth (Bulgakov, 2008). This is the probable reason for down regulation of proteins related to energy metabolism and protein synthesis in hairy roots. These all above studies are demonstrating the differences that exist between normal and hairy roots at gene level, protein level and also as in terms of antioxidant activities.