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Full Length Research Paper

Proteome analysis of *in vitro* and *in vivo* root tissue of *Withania somnifera*

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Proteomic analysis offers a new approach to identify a broad spectrum of genes that are expressed in living system. We applied this technique to investigate the protein changes under *in vitro* and *in vivo* conditions, since *in vitro* cultures is considered to be an alternative approach to traditional agriculture in the industrial production of the biomolecules. To better understand the proteins and enzymes involved in withanolide biosynthetic pathway, detailed two-dimensional gel electrophoresis (2-DE) and mass spectrometric analysis of *in vitro* grown adventitious roots and *in vivo* root samples of *Withania somnifera* were conducted. Of ~55 protein spots resolved in the two-dimensional gels, 35 protein spots were similarly expressed in both *in vitro* and *in vivo* root tissues and nine protein spots were differentially expressed only in *in vitro* root tissue and could be reproducibly displayed across an isoelectric focusing range of 4 to 7. A total of 44 protein spots (both *in vitro* and *in vivo*) were analyzed by matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF-MS). Homology search using MASCOT revealed high level of similarity in protein spots in both *in vitro* and *in vivo* root samples. This suggests that though *in vitro* roots are developed independent of shoot organs, they appear to have a similar developmental process as that of *in vivo* roots. This is the first report on establishment of a 2-DE reference proteome map of *Withania somnifera* roots and on the comparison of *in vitro* and *in vivo* root samples.

Key words: *Withania somnifera*, ashwagandha, *in vitro* roots, proteome, two dimensional electrophoresis, peptide mass fingerprinting.

INTRODUCTION

Ashwagandha (*Withania somnifera* Dunal, Solanaceae) is one of the most reputed Indian medicinal plants used for over 3000 years in ayurveda (Misra et al., 2005, Sangwan et al., 2004). Its ginseng- like health-promoting effects has earned it the popular name of Indian ginseng. Chemical constituents of *W. somnifera* are always of an interest for the researchers. The biologically active chemical constituents are alkaloids (ashwagandhine, cuswhygrine, anahygrine, tropine, etc), steroidal compounds, including ergostane type steroidal lactones, withaferine A, withanolides A-Y, withasomniferin-A, withanone, etc (Ganzera et al., 2003). Many pharmacological studies have been carried out to describe multiple biological properties of *W.somnifera* (Mishra et al., 2000). Pharmacologic activities of the plant include physiologic and metabolic restoration, antiarthritic, antiaging, nerve tonic, cognitive function improvement in geriatric states, and recovery from neurodegenerative disorders like convulsions and tardive dyskinesia (Bhattacharya et al., 2002; Dhuley, 2000). It

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has a significant role in prevention of various central nervous system (CNS) disorders, particularly in epilepsy, stress and neurodegenerative diseases such as Parkinson's and Alzheimer's disorders, tardive dyskinesia, cerebral ischemia, and even in the management of drug addiction (Kulkarni and Dhir, 2008). Preparations from roots and leaves have been traditionally used as tonic, hypnotic, sedative and diuretic. Owing to its adaptogenic effects, Ashwagandha products are available throughout the US as dietary supplements (Ganzera et al., 2003). *In vitro* and *in vivo* molecular pharmacologic investigations have elucidated the association of these activities with specific secondary metabolites known as withanolides present in the plant (Sangwan et al., 2007). As roots contain a number of therapeutically applicable withanolides, mass cultivation of roots *in vitro* will be an effective technique for the large scale production of these secondary metabolites (Murthy et al., 2008).

For commercial withanolide production, roots from field grown plant material are generally used. The quality of these products may be highly affected by different environmental conditions, pollutants and fungi, bacteria, viruses and insects, which can result in heavy loss in yield and alter the medicinal content of plant. Plant cell and organ cultures are promising technologies to obtain a constant supply of standardized plant-specific valuable metabolites (Verpoorte et al., 2002). Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cells/organs and to a condensed biosynthetic cycle. Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions and cells/organs can thus proliferate at higher growth rates than whole plant in cultivation (Rao and Ravishankar, 2002). Adventitious roots induced by *in vitro* methods have been reported to show a high rate of proliferation and active secondary metabolite accumulation (Hahn et al., 2003).

Studies have shown that any small change in the growth supplement or culture condition affected root growth *in vitro* and the accumulation of secondary metabolite thereof (Praveen and Murthy, 2010). The development of a fast growing root culture system would offer unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation (Murthy et al., 2008). Accumulation of secondary metabolite in *in vitro* root can be assured only if the developmental changes, protein profiling and metabolic profiling between *in vitro* and *in vivo* are clearly understood. Sharada et al. (2007) found that *in vitro* cultures established from leaves of *W. somnifera* accumulated the highest level of withanolides and those from shoot tips of well-grown plants produced the lowest level which suggested that tissue cultures having different morphology showed the inherent biosynthetic capability of the donor plant under *in vitro* conditions.

Proteomics has already been used for many different applications in plant sciences, including the study of proteins of biosynthetic pathways leading to secondary metabolites. Two-dimensional polyacrylamide gel electrophoresis (2-DE) is an established and powerful technique for analyzing the complex mixtures of protein. 2-DE along with matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses provide new insights into identification of biotic stress responsive protein in bread wheat (*Triticum aestivum* L.) and demonstrates the advantages of proteomic analysis (Kamal et al., 2010).

In secondary metabolism, many enzymes are involved, often working in close collaboration to catalyze cascades of reactions. Besides the enzymes, transport and regulatory proteins are also involved, which makes the proteome an essential topic for studying metabolic pathways. Until now no reports are available on the comparison of *in vitro* and *in vivo* roots in plants of commercial importance. Hence pioneering attempt has been made to study the profile of proteins expressed in *in vitro* and *in vivo* root samples of *W. somnifera.*

The overall goal of this project was to investigate the protein changes under *in vitro* and *in vivo* conditions. Separation of proteins in *in vitro* and *in vivo* root tissues of *W. somnifera* was done using 2-DE gels, and identification of similar and differentially expressed proteins was achieved using MALDI-TOF-MS. Of 55 total proteins resolved in 2-DE gels, only 26 were identified and found in available protein databases. Among those proteins, only one was found to differentially expressed in *in vitro* root tissue.

MATERIALS AND METHODS

Plant material

Two months old Murashige and Skoog (MS basal medium) (Murashige and Skoog, 1962) maintained leaves of *W. somnifera* plant were taken as explants. Adventitious roots were induced on MS media supplemented with three different combinations of auxin which included 1 mg/ml IBA (Indole 3-butyric acid) + 0.25 mg/ml IAA (Indole 3-acetic acid), 2 mg/ml IBA + 0.5 mg/ml IAA and 4 mg/ml IBA + 1 mg/ml IAA, among which roots were induced within 7 to 10 days on the media supplemented with 2 mg/ml IBA+0.5 mg/ml IAA. Those roots were transferred to MS basal media in suspension culture after a period of 5 to 10 days from induction. Fresh two months old *in vitro* roots were harvested and one year old *in vivo* roots (field grown) shown in Figure 1, were used for protein extraction immediately.

Sample preparation for 2-DE

Two months old *in vitro* and one year old *in vivo* roots of *W. somnifera* were used in this study. Tissues were ground thoroughly in liquid nitrogen and extraction was carried out using a modified procedure of Kim et al. (2003). Briefly, samples (10 g) were ground with liquid nitrogen and then incubated in sample buffer [50 mM Tris Base (pH 8.0), 10 mM NaCl, 1% sodium dodecyl sulfate (SDS)], 200 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at 100°C for 10 mins. The mixture was transferred onto ice. After centrifugation at 15 000 g for 30 min, the supernatant was

Figure 1. *Withania somnifera.* A, Whole plant; B, two months old *in vitro* roots; C, fresh *in vivo* roots.

precipitated with 10% TCA solution for 1 h at -20°C. The protein pellet obtained was washed with ice-cold acetone at least four times to remove contaminants followed by lyophilization. The pellets were dissolved in 0.5 ml of sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% DTT (dithiothreitol) and 0.5% IPG buffer (pH 4-7; GE Healthcare, Milan, Italy). After complete solubilization of protein pellets, the samples were centrifuged at 15000 rpm for 30 min at 4°C. The supernatant was collected and stored at -80°C until use. The protein concentration was estimated by Bradford protein assay kit (Bio-rod, Hercules, CA, USA) according to the manufacturer's instructions.

2-DE separation and protein visualization

2-DE was performed according to the IPG principles and methods of Amersham Biosciences (Piscataway, NJ, USA) with some modifications. In the first dimension, 13 cm IPG strips (pH 4-7) (Amersham Biosciences, Sweden) were rehydrated overnight with 250 µl of IPG rehydration buffer at room temperature. 150 µg of proteins were loaded to an IPG strips and isoelectric focusing (IEF) was conducted at 20°C with a Pharmacia Multiphor II separation unit (Amersham Biosciences, Sweden). A total run of 15 h was performed as follows, 50 V for 1 h, followed by 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 1 h, 8000 V for 8 h, and finally 50 V for 2 h. The focused strips were equilibrated twice for 15 min each time, first in 10 mg/ml DTT and then in 40 mg/ml iodoacetamide prepared in equilibration buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS. Proteins were separated in the second dimension according to their molecular weight using 12% SDS-PAGE. First dimension strips were attached to the second dimension gel with a 0.5% low melting point agarose solution (Amersham Biosciences). Gels were run at a constant 20 mA until the bromophenol dye front reached the end of the gel. The protein spots in analytical gels were visualized by silver staining (Shevchenko et al., 1996). Proteins of each sample were separated in triplicate.

***In situ* digestion of proteins**

Selected protein spots were excised manually from the 2-DE gel and protein digestion on the gels was performed as described by Shevchenko et al. (1996) with slight modifications. Briefly, the excised gel pieces were washed with 100 µl of 100 mM NH_4HCO_3 for 5 min and then dehydrated in 100 µl of acetonitrile for 10 min. 100 µl of reduction solution (10 mM dithiothreitol in 100 mM NH_4HCO_3) was added and incubated for 30 min at room temperature. After removing reduction solution completely, 100 µl of alkylation solution (100 mM iodoacetamide in 100 mM NH_4HCO_3) was added and incubated at room temperature in the dark for 30 min. Then the gel pieces were washed with 100 µl of 100 mM NH_4HCO_3 for 5 min and dehydrated with 100 µl of acetonitrile for 10 min. After drying under vacuum centrifuge, the gel pieces were rehydrated in 5 to 10 µl of 50 mM NH_4HCO_3 containing 20 ng/µl trypsin (Promega, Madison, WI, USA) on ice. After 45 min, the trypsin solution was removed and replaced with 10 to 20 µl of 50 mM NH_4HCO_3 without trypsin, and digestion was carried out for a minimum of 16 h at 37°C. The resulting tryptic peptides were subsequently extracted by the addition of 10 µl of extraction buffer followed by addition of 10 to 15 µl of acetonitrile. Pooled extracts were dried using a Speed Vacuum.

Protein dentification by MALDI-TOF-MS

Matrix-assisted laser desorption/ionization time-of-flight experiments were performed on a Voyager- DE STR mass spectrometer (Applied Biosystems, Framingham, MA, USA). Following digestion, the extracts were re-dissolved in 1 µl of extraction buffer and 1 µl of matrix solution (α-acyano- 4-hydroxycinnamic acid, HCCA), targeted onto the matrix-assisted laser desorption/ionization (MALDI) plate, was allowed to dry completely and then analyzed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and spectra were recorded.

Homology search

Matching of the experimental results with both theoretical digests and sequence information obtained from publicly available protein database was performed using the Mascot software, freely available at http://www.Matrixscience.com. Initial search parameters were as follows: carbamidomethyl cysteine as a fixed modification and oxidation of methionine as a variable modification, one missed cleavage site, peptide mass tolerance of ±50 ppm, and protein mass of 20 KDa. Database mass spectrometry Database (MSDB) and taxonomy Viridiplantae (Green plants) was used. The protein score is −10*log (P), where P is the probability that the observed match is a random event.

Table 1. Protein estimation for *in vitro* and *in vivo* root tissues of *W. somnifera.*

S/N	Sample	Protein concentration (mg/g)
1	*In vitro* root of *Withania somnifera*	1.3±0.11
2	*In vivo* root of *Withania somnifera*	2.4±0.13

*Data represents four replicates. Values are mean±S.E.

RESULTS AND DISCUSSION

Comparative 2-DE protein profile of *in vitro* and *in vivo* roots of *Withania somnifera*

In order to perform the 2-DE, whole proteins were extracted from *in vitro* and *in vivo* roots of *W. somnifera* using TCA precipitation. The amount of protein was estimated using Bradford's method (Bradford, 1976). The result is presented as a Supplementary Table 1. Protein content in *in vivo* root tissue was 2.4 mg/g, which was comparatively higher than that in *in vitro* root tissues (1.3 mg/g). Though a two-fold change in protein content was observed, the concentration was equalized for further analysis. The report of Cormack et al. (2001) suggested that roots record low amount of proteins compare to leaves and seeds. Generally, 18% proteins were profiled in leaves whereas roots have less than 0.25% protein in *Arabidopsis thaliana* root. The quantity of the protein content in the roots of *W. somnifera* was analyzed and it was observed that young roots contain low amount of protein compared to leaves and seeds (Khanna et al., 2006).

Two dimensional protein separation systems were employed to differentiate the proteins present in the extracts. The analysis was performed four times, and the spots that showed a high level of expression consistently were selected for analysis. The analysis showed a similar pattern of protein bands in both the extracts, suggesting the presence of complex mixture of different proteins in the samples. The 2-DE protein profile of *W. somnifera* roots was analyzed in 4 to 7 pH range and proteins were detected by silver staining (Amershan Biosciences).

Comparative analysis of 2-D gels revealed high level of similarity in the protein pattern of both *in vitro* and *in vivo* root sample. 35 spots were commonly present in the *in vitro* and *in vivo* root samples. Interestingly, nine (A to H, ISP (*in vitro* specific protein) differential spots were present in the *in vitro* root that were not found in the *in vivo* root (Figure 2).

Identification of separated proteins by MALDI-TOF MS

The individual spots were excised from the 2-D gel and digested with trypsin. The resulting peptide fragments were extracted and the mass of each was measured using MALDI-TOF MS analysis. Mass spectral data obtained was then subjected to MASCOT analysis. Tables 2 and 3 show the results of MASCOT analysis suggesting that, among the 35 protein spots analysed, 22 showed significant matching against MSDB. Among the nine (A to H, ISP) differential protein spots, only four showed significant matching against MSDB and among the four significant matches, three showed similarity with that of the similar spots. Number of matched peptides was between 3 to 6 with a maximum of up to 63% sequence coverage. Six spots (spots 1, 6, 15, 24, E, B) showed a good correlation between the experimental and theoretical isoelectric point (pI). Only one spot (spot 11) showed good correlation between experimental and theoretical molecular mass. Some of the identified proteins were present in more than one spot (e.g. spots 31 and 33, spot 24 and E). This could arise from several causes. One of them is post-translational protein modifications (PTMs), the other could be proteolytic degradation of the protein, or the reason that these proteins are products of different genes (Jorge et al., 2005).

Functional analysis of proteins expressed in both *in vitro* and *in vivo* root sample

The similar protein spots expressed in both *in vitro* and *in vivo* root samples identified from MASCOT analysis were categorized according to their biological functions: general cell metabolism, defense related proteins and secondary metabolite production (Table 2). Among the 35 protein spots analyzed, only 22 spots showed matches against the database, 12 protein spots were involved in general cell metabolism in which spots 2, 20 and 22 were identified as Probable U3 small nucleolar RNA-associated protein 11, 6 protein spots involved in defense mechanism in which spot 31, 33 were identified as ATPase. Four protein spots were involved in a secondary metabolite production in which all the three were identified as 12-oxophytodienoate reductase.

Proteins involved in general cell metabolism

In metabolism, some substances are broken down in the presence of enzymes to yield energy for vital processes while other substances, necessary for life, are

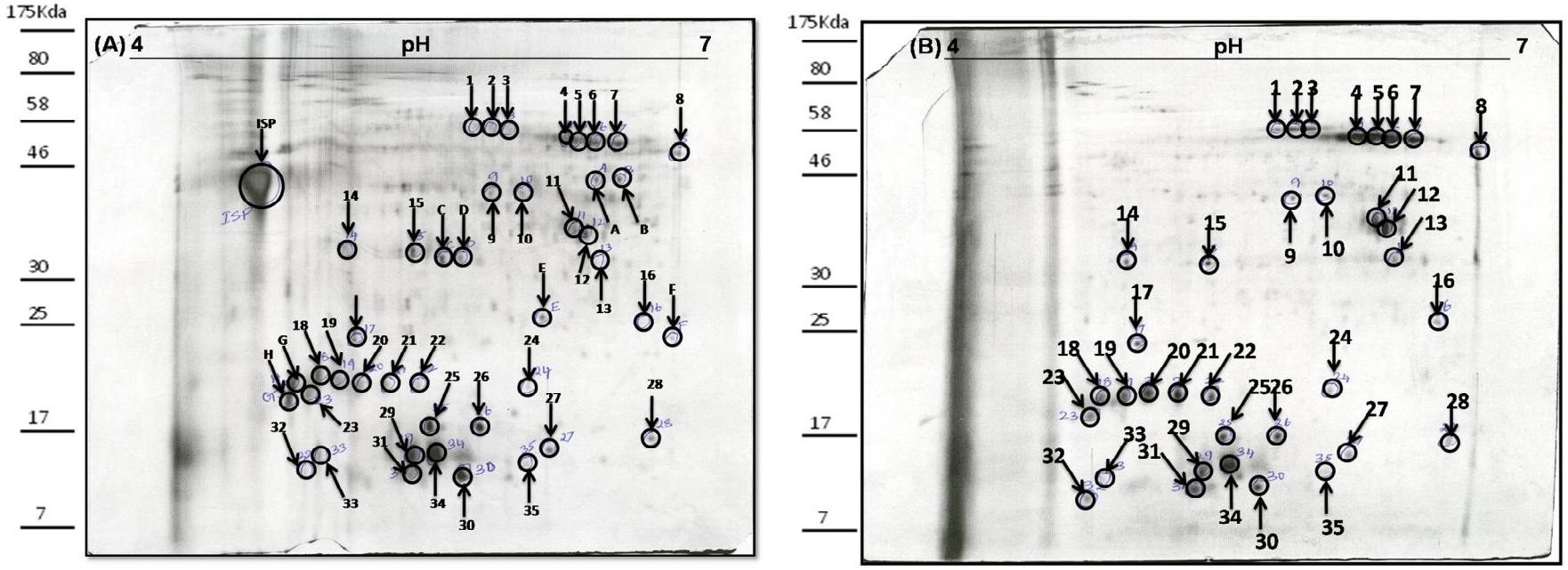

Figure 2. Comparison of 2-DE protein patterns of the *in vitro* and *in vivo* roots of *W. somnifera.* A, i*n vitro* root sample, showing 35 similar spots and differential spots viz A to H, ISP; B, *in vivo* root sample, showing only 35 similar spots.

synthesized (examples are DNA, RNA, and protein synthesis). The proteins identified in general cell metabolism category (energy production, RNA processing, protein synthesis, cell differentiation and signal transduction) are discussed; spot 24 was predicted as fructose-2, 6-bisphosphatase. Fructose-2, 6-bisphosphatase catalyzes the synthesis and degradation of fructose 2, 6 -bisphosphate (Fru-2, 6-P2), which is a powerful activator of 6-phosphofructo-1-kinase, the rate-limiting enzyme of glycolysis (Atsumi et al., 2005). Spot 7 was predicted as lysyl-tRNA synthetase, spot 34 was predicted as ribosomal protein small subunit 4 and spot 8 was predicted as 60S ribosomal protein L9; involved in protein synthesis and processing (Journet et al., 2002). Spot 2, 20 and 22 was matched with probable U3 small nucleolar RNA-associated protein 11 involved in RNA processing and modification (Kufel et al., 2000). Spot 18 was matched with U6 snRNA-associated Sm-like protein involved in RNA processing (Moore et al., 1993). Spot 25 was identified as maturase K. Maturase K are the splicing factors for the plant group II introns from premature RNAs (Mohr and Lambowitz, 2003).

Spot 11 was matched with proline-rich protein-2, which plays an important role in the differentiation and function of particular cell types (Ye et al., 1991). Additionally, proline is also utilized for protein synthesis, and large part of hydroxyproline, a derivative of proline through hydroxylation, is found in structural proteins, such as collagen in animals or hydroxyproline rich protein in plants (Hall and Cannon, 2002; Myllyharju, 2003). Spot 5 was identified as calcium ATPase (*Zea mays*), which are involved in the transduction of gravitational stimuli. On gravitational stimuli there is an increase in cytosolic-free Ca^{2+} levels, which in turn triggers a signal transduction cascade resulting in a physiological response (Urbina et al., 2006).

Proteins involved in defense mechanism

Plants possess chemical and physical defenses to reduce damage by primary consumers: animals and microbes that use them as a food source

Table 2. List of similar proteins expressed in both *in vitro* and *in vivo* root samples of *W. somnifera* which is categorized according to their biological function as general cell metabolism, defense mechanism and secondary metabolism.

Spot	Identified protein	Accession number (swissprot)	EMBL id	Matched Peptide	Score	Molecular mass (kDa) theoretical /experiment	pI theoretical/ experiment	Sequence coverage (%)
General cell metabolism								
24	6-phosphofructo-2-kinase (*Zea mays*)	O24559	AF007582 .1	5	63	43.2/20.5	6.15/6.1	15
9	Putative retroelement (*Oryza sativa*)	Q94I24	AC022352 .5	4	51	131.3/42	8.12/5.9	3
Protein synthesis								
7	Lysyl-tRNA synthetase (*Oryza sativa*)	Q10HI8	DP000009 .2	5	51	68.1/52	5.9/6.7	8
8	60S ribosomal protein L9 (*Pisum sativum*)	P30707	X65155 .1	4	42	22.1/50.5	9.21/7	32
34	Ribosomal protein small subunit 4 (*Dicranoweisia crispula*)	Q7YK70	AJ554014 .1	6	56	21.7/14.5	10.36/5.6	44
RNA processing								
2	Probable U3 small nucleolar RNA-associated protein 11 (*Oryza sativa* subsp. *japonica*)	Q8S1Z1	AP003260 .5	5	50	27.5/57	10.03/5.9	29
18	U6 snRNA-associated Sm-like protein (*Arabidopsis thaliana*)	Q8LCS5	AY086430 .1	3	41	9.9/21.5	7.82/4.9	30
20	Probable U3 small nucleolar RNA-associated protein 11	Q8S1Z1	AP003260 .5	4	41	27.5/20.8	10.03/5.1	24
22	Probable U3 small nucleolar RNA-associated protein 11	Q8S1Z1	AP003260 .5	5	53	27.5/20.8	10.03/5.4	24
25	Maturase K (*Mandragora officinarum*)	Q70D04	AJ585883 .1	4	37	60.6/17.5	9.64/5.5	10
Cell differentiation								
11	Proline-rich protein-2 (*Gossypium hirsutum*)	Q94G50	AF277674 .1	3	32	35.5/38	9.68/6.4	17
5	Calcium ATPase (*Zea mays*)	Q9LM01	AF096871 .1	4	32	113.5/53	7.00/6.4	3
Defense mechanism								
1	Soluble inorganic pyrophosphatase 1, (Arabidopsis thaliana)	Q9LXC9	AY551439 .1	4	34	33.6/57	5.71/5.8	16
6	Major allergen Cor a 1.- Corylus avellana (*European hazel*)	Q39454	Z72440 .1	3	32	17.7/52	6.10/6.55	31
15	Alcohol dehydrogenase (*Hordeum spontaneum*)	Q9FPF5	AF326704 .1	3	32	33.9/35	5.56/5.4	13
28	Cinnamyl-alcohol dehydrogenase (Medicago sativa)	Q53X16	L46856 .1	5	49	39.5/16	7.15/6.85	14
31	ATPase 1, plasma membrane-type (*Arabidopsis thaliana*)	P20649	M24107 .1	3	37	104.6/12.3	6.25/5.3	4
33	ATPase 1, plasma membrane-type (*Arabidopsis thaliana*)	P20649	M24107 .1	3	35	61.5/14.5	6.30/4.9	4
Secondary metabolism								
3	12-oxophytodienoate reductase 1 (*Solanum lycopersicum*)	Q9XG54	AJ242551 .1	5	42	42.8/57	5.86/6.05	13
16	F-box family protein, putative(*Oryza sativa*)	Q8LNJ4	AC079029 .11	5	52	35.09/26.2	9.09/6.8	16
19	12-oxophytodienoate reductase 1, LeOPR1	Q9XG54	AJ242551 .1	4	52	42.8/21.2	5.86/4.95	13
21	12-oxophytodienoate reductase 1	Q9XG54	AJ242551 .1	5	44	42.8/20.8	5.86/5.25	13

Table 3. List of differentially expressed proteins in *in vitro* root tissue of *W. somnifera* which is categorized as general cell metabolism and nucleic acid binding.

Spot	Identified protein	Accession number (swissprot)	EMBL id	Matched peptide	Score	Molecular mass (kDa) theoret/ experiment	pI theoret/ experiment	Sequence coverage (%)	Category
E*	6-phosphofructo-2-kinase (Zea mays)	O24559	AF007582 .1	4	46	43.4/26.3	6.15/6.15	12	General cell metabolism
B	Poly(A)-binding protein (*Triticum aestivum*)	P93616	U81318 .1	4	48	71.1/44	6.60/6.7	7	Nucleic acid binding
C*	MaturaseK (*Trochocarpa laurina*)	Q9BAA0	AY005106 .1	5	51	58.9/34	9.78/5.65	12	General cell metabolism
G*	U6 snRNA-associated Sm-like protein (*Arabidopsis thaliana*)	Q8LCS5	AY086430 .1	4	54	9.9/21	7.82/4.6	50	General cell metabolism

***** These spots overlap with that of the similar spots, expressed in both *in vitro* and *in vivo* root tissues of *W. somnifera*.

(Heil and Ton, 2008). In response to any kind of stress, plant produces many defensive proteins, enzymes and secondary metabolites which inturn triggers signal transduction thereby protecting plant from the damage. Some of the identified defence related proteins are soluble inorganic pyrophosphatase, ATPase and alcohol dehydrogenase which are involved in nutrient stress and survival during flooding.

Spot 1 was predicted as soluble inorganic pyrophosphatases 1(PPase). PPase are important enzymes that catalyze the hydrolysis of inorganic pyrophosphate (PPi) to inorganic phosphate (Pi). They participate in the assimilation of mineral nutrients (Jardin et al., 1995; George et al., 2010) which suggests that PPi metabolism might be indirectly involved in mediating drought stress responses in *Nicotiana benthamiana* leaves. Spot 15 was predicted as alcohol dehydrogenase. Alcohol dehydrogenase (ADH) is involved in the ethanolic fermentation pathway that is responsible for the reduction of acetylaldehyde which is toxic to plant tissues, to ethanol resulting in continuous regeneration of NADP in the cytoplasm (Chung and Ferl, 1999). Hence, induction of ADH can enhance survival of plants under flooded conditions (Johnson et al., 1994). Spot 31 and 33 was predicted as ATPase, which is involved in nutrient and environmental stresses (Shen et al., 2006). Spot 28 was predicted as 6-Cinnamyl-alcohol dehydrogenase, which is a key enzyme in lignin biosynthesis as it catalyzes the final step in the synthesis of monolignols. Lignin is a phenolic heteropolymer in secondary cell walls that plays a major role in the development of plants and their defense against pathogens (Barakat et al., 2009).

Proteins involved in secondary metabolism

Spots 3, 19 and 21 were predicted as 12-oxophytodienoate reductase 1, involved in the biosynthesis of jasmonic acid, a potent signal molecule in the defense system of plants, where it regulates the expression of many wound-activated and defense-related genes (Breithaupt et al., 2001). Jasmonic acid (JA) is an important phyto-hormone that regulates plant defense responses against herbivore attack, pathogen infection and mechanical wounding (Bu et al., 2008). Spot 16 was predicted as F-box family protein, which is an important component of the E3 ubiquitin ligase Skp1-Cullin-F-boxprotein complex. It binds specific substrates for ubiquitin-mediated proteo-lysis. F-box proteins act as important receptors and signaling components in plant hormone signaling pathways (Yu et al., 2007).

Functional analysis of proteins that were expressed differentially in *in vitro* root sample

Under *in vitro* conditions, the optimum nutrient concentration, temperature and time period in culture are the critical determinants in controlling the growth and stability of adventitious roots, accumulation of secondary metabolite and also the protein expression pattern (Praveen and Murthy, 2010). Mass production of root can be obtained from *in vitro* cultures. Even though *in vitro* cultures are maintained under controlled conditions, the 2-DE protein profile was significantly similar to that of the field grown plants or *in vivo* plants. The MASCOT result obtained for *in vitro* root samples seems to be similar with that of the results of the *in vivo* root sample. Proteins expressed in *in vitro* root sample are presented in Table 3. They are involve in general cell metabolism and nucleic acid binding. Among the nine protein spots analyzed, protein spots E, C and G (fructose-2, 6-bisphosphatase, maturase K and U6 snRNA-associated Sm-like protein) are involve in general cell metabolism; overlaps with that of the similar spots which reveals that protein

expression pattern of both *in vitro* and *in vivo* root samples are almost similar and their general cell metabolism is not affected even though there is a difference in environment and culture conditions. Poly (A)-binding protein (spot B) (PABP) is the only differentially expressed protein in the *in vitro* root sample which was revealed by MASCOT analysis. It is a ubiquitous, essential factor involved in mRNA biogenesis, translation, and turnover. PABP participates in at least three major post-transcriptional processes: initiation of protein synthesis, mRNA turnover, and mRNA biogenesis (Belostotsky, 2003). PABP also plays a complex role in mRNA degradation, inhibits mRNA deadenylation, as well as decapping (Bernstein et al., 1989; Caponigro and Parker, 1995; Wilusz et al., 2001).

To our knowledge, this study demonstrates for the first time the comparative proteome analysis of *in vitro* and *in vivo* root samples of *W. somnifera* using 2-DE, and MALDI – TOF/MS. The results indicate that the expression pattern and the protein identified in both *in vitro* and *in vivo* roots of *W. somnifera* are similar, in spite of providing exogenous plant growth regulators for the *in vitro* root induction. Though, proteome analysis specific secondary metabolism related proteins involved in withanolide synthesis were not identified but differences in the accumulation of secondary metabolites were observed in the thin layer chromatography (TLC) pattern between the *in vitro* and *in vivo* roots analyzed (unpublished data) revealing that there must be some changes in the protein profiling of *in vitro* root in terms of secondary metabolite related proteins which needs further analysis to confirm these results. The possible reason may be that, *in vitro* roots are grown devoid of any shoot tissue and hence, any metabolite synthesized will either be accumulated in the roots or be secreted into the medium. As no significant secretions were observed in the medium, it is assumed that, all metabolites produced are accumulated in the root tissues. The sensitivity of 2D profiling should be increased and more spots should be analyzed in order to understand the discrepancy observed in the results analyzed by the two different techniques. Thereby enhancing the protocol will provide more information regarding the secondary metabolism. This study has to be carried out for the root samples at various time periods and proteomics data can be compared with that of the EST (Expressed sequence Tags) data, which would provide more information on secondary metabolite synthesis.

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