Plant Omics Journal POJ

POJ 5(2):167-176 (2012) ISSN:1836-3644

Review article

Proteomics of nitrogen fixing nodules under various environmental stresses

Sowbiya Muneer[1,2], Javed Ahmad[1], Humayra Bashir[1] and M. Irfan Qureshi[1]*

[1]Proteomics and Bioinformatics Lab, Department of Biotechnology, Jamia Millia Islamia, New Delhi, 110025, India
[2]Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture & Life Science, Chonnam National University, Buk-Gwangju P.O Box 205, Gwangju, 500-600, Korea

***Corresponding author: mirfanq@gmail.com**

Abstract

Proteomics is an ideal tool to study the interaction of root nodules and their symbiotic bacteria as it provides a broad overview of proteins produced by both partners during their constant signal exchange and allows the signal transduction path ways following photophosphorylation. Iron containing proteins play a key role in symbiotic nitrogen fixation that occurs in a nodule-a specialized structure present on roots. Several proteins like those related to SNF (symbiotic nitrogen fixation), predominantly components of nitrogenase complexes, such as *nif*D, *nif*H, *nif*K, nitrogen regulatory protein II (GlnB) and PIIA (PtsN), and urease accessory protein (UreE) have been found to be affected by abiotic stress. Nodules are better equipped with all kinds of antioxidant systems (i.e., ascorbate-glutathione pathway or Superoxide dismutase) which have been formed to show a decline under stress conditions. The present review article aims to investigate the nodule physiology, the effect of different abiotic stress on nodule proteins comprehensive account of these stress-responsive proteins and their role in combating stress in legume nodules. This will help to elucidate which specific key proteins are affected by abiotic stress. As such, it will greatly facilitate understanding resistance or stress tolerance mechanism and hence improvement in crop resistance.

Keywords: abiotic stress, mass spectrometry, nodule physiology, nitrogen fixation.
Abbreviations: SNF (symbiotic nitrogen fixation), IPG (immobilized pressure gradient), SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), MALDI-TOF (matrix assisted laser detection ionization time of flight), MS (mass spectrometer), ESI (electrospray ionization), PMF (peptide mass fingerprinting), HSP (heat shock proteins).

Introduction

Nitrogen is the mineral nutrient needed in greatest abundance by plants. Atmospheric nitrogen is reduced to ammonia, to become available to plants by several processes (Beringer et al., 1980). One of the most important modes of N_2-fixation is biological symbiotic N_2-fixation which takes place in presence of nitrogenase, a highly conserved enzyme comprised of proteins like *nifK*, *nifD* and *nifH*. There is a large group of leguminous plants that harbor N_2-fixing machinery in their legumes (Willey et al., 2008). Legumes are thus very important plant organ where N_2-fixing bacteria interact with host plant. The efficiency of nodules in terms of N_2-fixation is influenced by biotic and abiotic factors/stress. In order to study the protein-protein interactions of bacteria, fungi, and plants, proteomics is an ideal tool. The use of proteomic techniques helps us to study the nodule protein profile and how it is influenced by different biotic and abiotic stress. The existing literature reveals that proteins involved in defense mechanism against biotic and abiotic stress accounted for 12% of all identified proteins in nodules. These proteins protect plants against pathogenic fungi, bacteria, viruses, and adverse environmental conditions, which have been found to be induced by a variety of biotic and abiotic stimuli such as wounding, pathogen, infection (Kav et al., 2007) and environmental abiotic stress. Various proteins involved in nitrogen fixation have been found to be up-regulated or down-regulated. Under normal growth conditions, proteins were identified and categorized in *Rhodospirillum ruburm* (Selao et al., 2008) by functional categorization and differential expression compared to nitrogen fixation. Since the nodule proteomics has not been well documented, we focused on plant microbe interaction, nodule physiology and proteomic studies of nodules under various environmental stresses.

Plant-microbe interaction

The vast majority of plant-microbe interactions are no way to surprise that many have evolved close relationships; many such plant-microbe interactions benefit the plants. Symbiotic microorganisms can associate physically with other organisms in a variety of ways. One organism can be located

on the surface of other as an ectosymbiont and other organisms can be located beneath the surface as an endosymbiont. The most important example for plant-microbe interactions is seen in process of nitrogen fixation which actually is an endo-symbiotic association between roots of leguminous plants and bacteria converting enzymatically gaseous nitrogen (N_2) to nitrate and nitrite. This relation is also accompanied by mycorrhizal fungi which contribute to plant phosphorus acquisition. There is a specific mechanism in legumes for nitrogen fixation. The plant roots secret a number of compounds among which a compound called "flavonoid" that stimulates rhizobial colonization of root surfaces, these flavonoid bind to a bacterial protein to form different kinds of nitrogen fixing genes including nod genes. The nod genes are expressed specifically in response to plant-produced flavonoid compounds. Central to the regulation of the nod genes is NodD, a LysR-type regulator, which activates nod gene expression only in the presence of the flavonoid inducer (Loh and Stacey 2003). The proteins which are responsible for the formation of nodules are called nodulins encoded by nodulin gene. The rhizobia forms a specific factor on host called Nod factor. These lipochito-oligosaccharide, bind to a specific receptor kinase of the host which are a part of signal transduction pathways. The process of nitrogen fixation takes place in presence of enzyme called nitrogenase. Nitrogenase is a very sensitive enzyme hence nitrogen fixation takes place only when concentration of oxygen is low because oxygen which is diffused together with nitrogen in nodules is consumed by respiratory chain which is present in bacterial membrane. Due to a very high affinity of bacteroid cytochrome-ala3 complex, respiration is still possible at very low concentration of oxygen. At least 16 ATP molecules are used to fix one molecule of oxygen. The outer layer of nodule is a diffusion barrier for the entry of air. The diffusion resistance is so high that bacterial respiration is limited by the uptake of oxygen. Moreover it serves as a buffer in the host plant. Legume plants are special being successful to establish a symbiotic relationship with nitrogen fixing bacteria, such as genus rhizobium and related probacteria. Thus N_2 fixing bacteria in association with their leguminous host plants have been the subject of intensive investigation with a variety of approaches and methodology. Under stress conditions, the plants have an outburst of oxidative stress producing a mixture of superoxide radicals, hydrogen peroxide, and N_2O along with other types of responses. The legume nodules operate various antioxidant mechanisms including ascorbate-glutathione cycle and other defense mechanisms in order to mitigate the oxidative stress helping to maintain metabolic pathways near normal.

Structure and Composition of nodule

The formation of nodule (Fig 1) and its structural feature (Fig 2) has been studied in many leguminous plants. In many legumes, the first step in nodule formation involves rhizobial induced deformation of host root hairs. Initially it was thought that nodules of legumes were caused by plant disease, until their function in nitrogen fixation was recognized by Hellriegel and Wilfarth in 1888. They found that beans containing these nodules were able to grow without nitrogen fertilizer. The rhizobia form species-specific nodulation factors (Nod factors). These lipochito-oligosaccharide acquire a high structural specificity (e.g. by acylation, acetylation and sulfatation). They are like a securely key with many notches and open the house of the specific host with the rhizobia associate. The mechanism of entry is obscure; they suggest that the enzyme polygalacto-uranase is implicated. This enzyme, induced in susceptible plants by a water-soluble substance produced by the appropriate rhizobia which allow the bacteria either to penetrate the root-hair wall or perhaps to produce an elongated invagination of the root hair surface. The invagination hypothesis, advanced by Nutman 1956, 1957; is an attempt to explain the formation of the so called "infection thread," a tube which encases the bacteria within the root hair and which seemingly carries the microorganisms into the root cortex. These threads have been observed by Ward (1887) who considered them to be fungal hyphae, but their nature became evident after Beijerinck (1888) demonstrated the bacterial nature of the causative agent of nodule formation. Early workers (Allen et al., 1958) considered the infection threads to be bacteria enclosed within strands of slime; however earlier McCoy (1932) observed that the mucoid thread was enclosed within a sheath having the same general composition (cellulose, hemicelluloses, and perhaps certain pectic substances) as young plant cell walls. She noted that the sheath was always absent from the thread as it crossed the middle lamella during its passage from one plant cell to another and that the point of origin of the sheath was the host wall itself. Later, Schaede (1941) confirmed the presence of the cellulose sheath by the use of polarized light. A funnel-shaped formation, produced where infection thread and host cell wall join, has been observed by many of the early investigators, and is produced by a widening of the thread. It has been attributed to a variety of causes (Allen et al., 1958) including an impeded passage of the rhizobia into the cell from the middle lamella and physical effects of host walls expansion. Thorton (1930) stated that the end of the infection thread was open, exposing the zoogloeal mass within, and that bacteria could be detached from this mass and pass into the host cell cytoplasm. The same author also observed that swellings occurred along the sides of the thread and that these could ultimately burst, thus discharging the bacteria; this concept was previously elaborated by Dangeard (1926) and accepted by Lechtova-Trnka (1931). Ward (1887) and Beijerinck (1888) made careful drawings of such vesicles. In endomycorrhiza the fungi live within the root cortical cells. The fungus grows intercellularly but does not form Hartig net and mantle like those of ectomycorrhiza. Several other types of mycorrhiza have been identified in host pathogen interaction like arbuscular mycorrhizae which have association with other tropical plants and importantly with crops plants. These microbes enter root cells between the plant cell wall and invagination in plasma membrane. Although they belong to endomycorrhiza, they do not breach the root cell membrane they form a tree like invagination called arbuscules in plasma membrane. The nitrogen fixing bacteria are also associated with mycorrhizal fungi; a mycorhizsphere is formed by the flow of carbon from plants into mycorrhizal fungi. Bacterial symbionts have been found in cytoplasm of arbus mycorrhizal fungi (AM) which help in nitrogen metabolism of plant fungal complex by assisting with the synthesis of essential amino acids.

Proteomics of mycorrhizal and symbiotic association

Proteomics has become an important tool to study of plant biology towards mycorrhizal and symbiotic association (Hazelwood 2003; Kersten et al., 2002; Rossignol et al.,

Table 1. Proteins identified as a result of differentiated expression under different abiotic stress analyzed by MALDI-TOF MS/MS.

S. No.	Name of Protein	References
1	Drought Stress 19 KD globulin precursor, Glutelin type 1 Precursor, Nucleotide diphosphate kinase, B1160F02.9, CPN 21,	Ceser et al., 2009; Larrainzar et al 2007; Jorge et al., 2006
2	Salt Stress Cytochrome P450 71D9, ATP synthetase subunit beta, Inositol 3 phosphate, Ferredoxin-dependent glutamate	Veeranagamallaiah et al., 2008
3	Temperature HspH, HspD, HspB, GroEs1, GroES2, Hsp 83, Hsp 17, Hsp 16.9B, Hsp 17.9B	Sule et al., 2004; Munchbach et al., 1999
4	Cadmium Stress Cytochrome P450, Putative ribosomal protein, Cytokinin oxidase, ABC transpoter like protein, AAA ATPase family,	Sule et al., 2004
5	Plant pathogens Leghemoglobin I (92 %), NifH, nitrogenase reductase, LIR18B Protein, Ascorbate peroxidase, 60-KDa chaperonin, GroEL C, Elongation factor TU	Siria et al., 2000
6	Nitrogen metabolism Glutamine synthetase, glutamate ammonia lyase, a-glutamyl cysteinesynthase, aspartate aminotransferase, glutamate decarboxylase1, glutamate dehydrogenase, formate dehydrogenase, carbamoylphosphate synthetase, ferridoxin-nitrite reductase, nitrilase, UTP-ammonia lyase, glutamyl-tRNA synthetase/ligase, aspartyl-tRNA synthetase	Sarry et al., 2006

2001; Van Wijk et al., 2001; Thiellement et al., 1999). About 400 proteins have been identified with the development and functioning of both mycorrhizal and rhizobial symbiosis (Bestel-Corre et al., 2004; Rolfe et al., 2003; Trevaskis et al., 2002). Several unique symbiosis related proteins have been identified to show symbiotic association specifically Protein profile changes under different conditions and various life cycle phases as well as the organization of symbiotic association from ectomycorrhizal fungus *Tuber bronchii* has been identified by two dimensional gel electrophoresis (2D). In a symbiotic relationship of *T. bronchii* with angiosperms and gymnosperms few proteins have been identified like ubiquitin by N-terminal sequencing (Vallorani et al., 2000). In addition to this, some other proteins from mycorrhizal associations have also been identified with unknown functions (Fester et al., 2003; Benabdellah et al., 2000). The proteins from symbiotic association between *Glomus intracacies* and *Medicago trancatula* have also been identified from which three independent genotypes of rhizobium has been identified (Amiour et al., 2006). Further characterization of symbiotic association of *Medicago trancatula* and *Medicago alba* nodule specific proteins, nodule suppressed proteins, transporters, vitamin synthesis related proteins have been observed (Djordjevic et al., 2004). Sarma and Emerich (2006) identified proteins related to nitrogen fixation and enhanced expression of chaperons. Signal transduction genes have been induced in plant-microbe interactions, about twenty seven tentative consensus sequences (TC) having similarity to host disease resistance and defense response genes has been identified *in silco* analysis out of these eight shares protein sequences to putative plant disease resistance genes (R genes), six of them have been found clustered. Certain pathogen related proteins has been identified as a Pi49 protein, pathogen related proteins of class 10 (Pr10)-like have been identified in *M. trancatula* (Frank et al., 2004). A proline rich protein, a glycine rich protein and isoliquiritigenin 2-0-methyltransferase has been found up regulated, proteins like CAS18, a cold acclimation specific protein or dehydrins like protein has been detected as down regulated. Certain cDNA encoding MtN1 protein has been found up regulated in effective nodules in *M. trancatula* (Mesfin Tefaye et al., 2005). These proteins have been found a function not related to defense response in developing and fixation in nodules. Other nodule-enhanced genes from *in silco* and cDNA array analysis include cysteine protease and proteinase inhibitors. Kruger et al., (2002) and Xia et al., (2004) suggested that plant proteases are involved in host defense. With regard to signal transduction pathways, 43 genes have been found in *Lotus spp.* Approximately 31 TC assembled from 131 EST exclusively from *Sinorhizobium* inoculated tissues has shown homology to genes involved in signal transduction. A symbiotic relation between *Vigna unguiculata* and *Rhizobium sp.* has been analyzed by two dimensional gel electrophoresis (2D). Twelve symbiotic specific proteins have been identified and seemed to be associated with root-hair deformation and nodule development. Although proteomic characterization of *Sinorhizobium meliloti* bacteroid has been previously described (Djordjevic et al., 2004). About 97 proteins have been identified; several chaperonin proteins, including GroEL, GroEs and other heat shock proteins, which are essential to the establishment of symbiosis (Yeh et al., 2002) has also been detected. Some bacterial proteins related to chromosome-encoded serine hydroxymethyl transferase like GlyAl, SMc01770, glutamyl-tRNA aminotransferase (GatB, SMC01350), 50S ribosomal proteins L3 (RPlC; SMc01309) have been found to more abundant in drought stress. Mathesius (2001) used proteomic approach in *M. truncatula* to detect the AHL (N-acyl homoserine lactone) signals from pathogenic bacteria *S. meliloti* they observed 1/3 of the responsive proteins to the

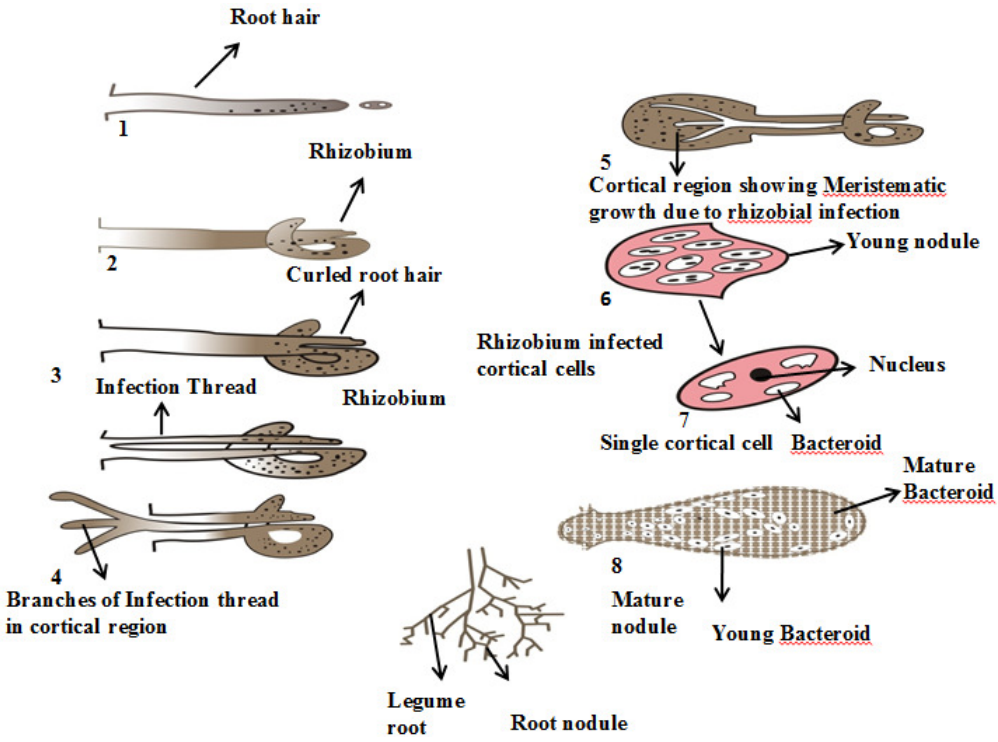

Fig 1. Formation and establishment of root nodule (1) Root hair (2) Establishment of rhizobium to root hair (3) Curled root hair (4) Infection thread by the infection of rhizobium (5) Branched infection thread in cortical region (6) Meristematic growth due to rhizobial infection (7) Formation of young nodule infected with rhizobium (8) Formation of bacteriods.

two AHL is distinct. The proteins have been analyzed by using 2D in *M. trancatula* after infection with *Aphanomyces euteiches* pathogen by Colditz et al (2004). A number of protein spots has been differentially observed and were found that they belong to the family of class 10 pathogenesis-related proteins and rest of the proteins were corresponding to the putative cell wall proteins and enzymes of the phenylpropanoid-isoflavonoid pathway. A similar study has been documented in *Pisum sativum* the number of proteins belonging to the carbohydrate and nitrogen metabolisms (Castillejo et al., 2004). In other studies, soybean has been used to identify proteins responding to pathogen invasion (Mithofer et al., 2002), salt stress (Aghaei et al., 2009), flooding (Shi et al., 2008), seed germination (Xu et al., 2008). Soybean peroxisomal proteins, xylem sap, and glycines have also been characterized by proteomics (Arai et al., 2008; Krishnan et al., 2007; Djordjevic et al., 2007; Natarajan et al., 2006, 2007).

Proteomics of nodules under abiotic stress

Proteomics is the study of proteins and their interaction is a cell, it is the protein equivalent to genomics that has attracted attention of bimolecular researchers worldwide. It encompasses a broad overview of certain parameters related to proteins such as its types, concentrations, protein-protein interaction etc (Qureshi et al., 2007; 2010). It is the best technique to study of plant-microbe interactions because it provides a broad range of proteins produced by partners i.e., roots, bacteria and fungi during their constant signal exchange. It also allows to study the signal transduction path-ways following photophosphorylation related changes in proteins which are important for protein functions (Qureshi et al., 2010). For mapping the proteome (total proteins at a given time) of nodules, the proteins are extracted from nodules and dissolved in non-denaturing detergents before being loaded on strips with an immobilized pH gradient (IPG) and subjected to an isoelectric focusing as a first dimensional run. The focused IPG strip is then reduced and alkylated and loaded onto the SDS gel (SDS-PAGE) for a second-dimensional run. Further, the gel is stained using an appropriate dye such as Coomassie Brilliant Blue stain or silver stain etc. Certain other stains are now commercially available for better identification of protein spots (Sypro dyes) between the gels to be compared. Using image analyses, under- and over expressed proteins is detected along with the proteins that are newly synthesized (or induced) and proteins that have disappeared. After identification of a set of differentially expressed spots from a series of two-dimensional gels by any image analysis (PD Quest, Progenesis, Decyder, Melanie etc.) software, the next step is tryptic digestion to identify the cognate proteins which can be achieved by MALDI-TOF MS/MS, ESI-MS, ion trap, etc. However, MS is now the method of choice for both protein identification and characterization. Mass spectrometers are coupled to protein database with advanced software that facilitate protein identification and structural analysis and to provide

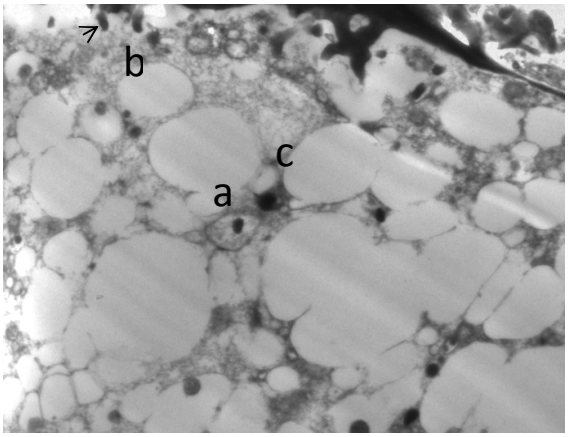

Fig 2. Structure and composition of root nodule in *Vigna radiata* (L.) (a) Vesicle (b) Small dense bacteria lying free within the host cell (c) Bacteriods embedded in cytoplasm.

an instant online bridge between mass spectra and public sequence databases (Aebersold et al., 2003; Ferguson et al., 2003; Lin et al., 2003). The first step towards protein identification is excision of two-dimensional gel plugs containing the protein spots of interest, in-gel digestion with minimal amount of protein a site-specific protease (commonly trypsin) and, finally, MS analysis of the resultant eluted peptides. Two MS platforms in particular represent powerful tools for proteomic studies. The first, MALDI-TOF MS, is typically used to measure the masses of the peptides derived from the trypsinised parent protein spot, generating a 'peptide mass fingerprint' (PMF) or *de novo* sequencing by ESI MS/MS. Software package then compares the peptide mass list with a predicted theoretical list of tryptic peptide fragments for every protein in the public databases, together with equivalent translated genomic and EST databases. In this case, the protein is identified based on the *in silico* match of experimentally determined versus predicted peptide masses, together in some cases with the apparent and predicted isoelectric point and molecular mass from the two-dimensional gels, rather the than actual amino acid sequence. Although each PMF is usually a viable means of assigning identity to a specific protein, as a result of the variability in amino acid sequences and of the relative distribution of protease cleavage sites between proteins (Godovac-Zimmermann et al., 2005), members of protein families with a high degree of sequence similarity can also result in effectively indistinguishable PMFs. This problem is exacerbated by the fact that it is unusual for the full complement of peptides for any given protein to be ionized and detected experimentally by MALDI-TOF. Different proteins have been found to be up-regulated under the corresponding abiotic stresses, so as to give a comparative overview with the proteins discussed earlier. Proteins associated with nitrogen and sulfur metabolism as detected using proteomic tools have also been mentioned. EST or protein database is identified by searching specific algorithms (Fig 3).

Stress responsive proteins under different abiotic stress

To study the changes in proteome under various biotic and abiotic stresses, several legume plants have been considered as a model plant which provided valuable information about the proteins. High throughput analysis of genes transcripts, proteins and metabolites have been applied to rhizobial and mycorrhizal symbiosis for high through put analysis of proteins 'proteomics' is employed (Bestel-Corre et al., 2004; Qureshi et al., 2010). In response to abiotic stresses namely drought, salt, temperature, heavy metal, pathogens and nutrients changes in protein profile have been reported. Proteins which are important in terms of comparative change either up-regulated or down-regulated have been established in Table 1.

Drought Stress

Drought is one of the environmental factors most affecting crop production. Under drought symbiotic nitrogen fixation is one of the physiological processes to first show stress responses in nodulated legumes. One of the most important legume crops to study the proteomic of N_2-fixing legumes under drought stress has been studied in *Medicago trancatula* (Bestel corre et al., 2002). Various bacterial proteins involved in N_2-fixing, proteomics have been used to identify them. About four proteins induced flavonoid elicitors in *R. leguminosarum*, NodB, NodE and other low molecular mass proteins with no homology to known proteins has been identified. Chen et al. (2000, 2005) identified 59 up-regulated and down-regulated proteins from nolR mutation having different functions like basic metabolism, heat shock, protein synthesis, translation, oxidative stress, and cell growth in *S. meliloti*. Other proteins like MyK15 have been identified in relation to abiotic stress (Fester et al., 2003). Furthermore proteins involved in signaling processes in nitrogen fixing nodules have been analyzed. Two calmodulin like proteins (CaML) has been identified corresponding to proteins CaML2 and 6b. A third protein CaML4 has also been detected in *M. trancatula* (Estı´baliz et al., 2007). Some other proteins related to SNF (symbiotic nitrogen fixation), predominantly the components of the nitrogenase complexes such as nifD, nifH and nifK and other nitrogen regulatory proteins PII (GlnB), PIIA (PtsN) and urease accessory proteins (UreE) has been identified in the root nodules which are important for the nitrogen fixation in legumes.

Salt Stress

Salt stress is one of the major environmental stresses conditions that adversely affect legume production in arid and semiarid regions, particularly because these plants take

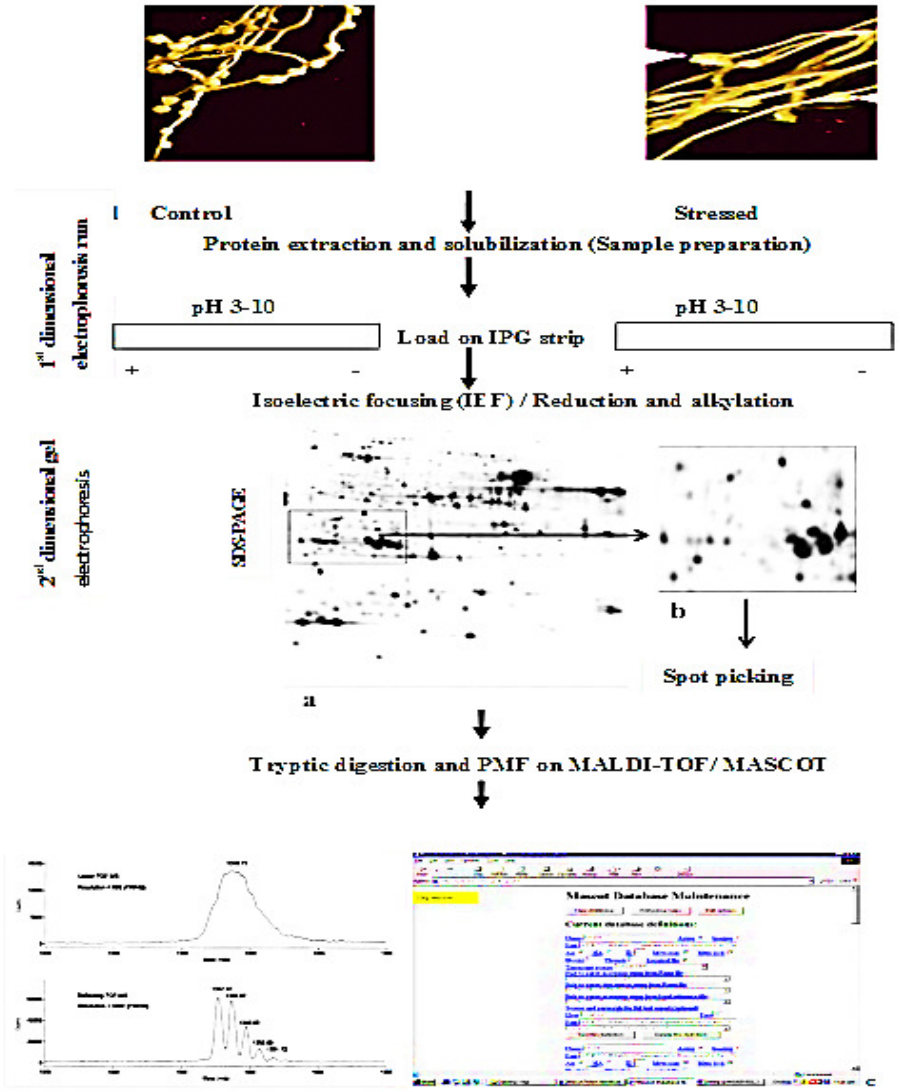

Fig 3. Demonstration of 2D gel electrophoresis (A) 2D gel, (B) corresponding to gel areas showing differentially expressed proteins and Excised to spot picking for tryptic digestion, (C) Mass spectrometry analysis and Protein spot identification by MASCOT.

most of their nitrogen demands from symbiotic N_2 fixation (El-Sheikh et al., 1995). Salt stress reduces the formation of nodules and leghemoglobin. The proteomic analysis towards salt stress has been analyzed on different leguminous plants. Up regulation and down regulation of proteins has been identified in *Rhizobium etli* and *S. meliloti*. Six proteins have been found to be over expressed. A protein of 65 KDa have been expressed and detected over expression of four more proteins. About two groups of proteins have been identified in *S. meliloti* strain. These proteins have been found to function as receptor for osmotic solutes, produced by bacterial cells. Another over expressed proteins identified have been found involved in biosynthesis of spermidin by decarboxylation of ornithine and arginine. Six other proteins have been found to be down regulated belonging to ABC transporters (Shamseldin et al., 2005). Some other proteins identified under salt stress have been found like conserved hypothetical protein, hypothetical protein SM21133, SME591787, putative oxidoreductase protein. A protein identified as putative oxidoreductase with possible function in cell envelope has been found down regulated. At least 47 proteins have been identified (Aghaei et al., 2009) under salt stress. Twenty three proteins were identified by N-terminal sequencing from which five proteins has been identified from their internal sequencing. It have been suggested that these new salt responsive proteins may play an important role in salt tolerance, salt stress differentially affects the expression of proteins in shoots. Photosynthesis-related proteins and those related to protein synthesis are markedly down-regulated, whereas osmotin-like proteins, HSP, calreticulin, and two new proteins have been markedly up-regulated in *Rhizobium etli* and *S. meliloti*..

Temperature stress

It is well known that legumes show adaptation to an unambiguous temperature. Legumes face both high and low temperature growing in tropical areas. The temperature below 10 ^{0}C comes under cold stress and the legumes grown at the temperature at which the cells freeze comes under freezing temperature 0 ^{0}C. The HSPs and their homologues must perform many essential functions in both normal and stressed cells. The current hypothesis is that HSP60, HSP70 and HSP90 function to alter the conformation or assembly of other protein structures. A number of stress protein families, including HSP90, HSP70, chaperonin 60, HSP40, the LMW stress proteins and ubiquitin, have been identified in diverse phyla (Lewis et al., 1999). Under normal conditions, several of the major stress proteins are present at low levels and function as 'molecular chaperones' key components contributing to cellular homoeostasis in cells under both optimal and adverse growth conditions (Wang et al., 2004). Several proteins under low and high temperature shows up regulation or down regulation of protein, nineteen proteins were observed to be induced, 12 *de novo* and seven others were clearly up regulated. Several other proteins identified under temperature stress in *B. japonicum* have been identified as heat shock proteins. The heat shock-induced proteins that have been identified fell into four classes: known sHsp, novel sHsp homologues, GroESL/DnaK proteins and unknowns. Of the 10 sHsp, two, HspA and F, have not been observed under temperature stress (Martin 1999). The proteomic analysis has also shown the presence of YufN protein which is a member of the ABC transporter family embedded in halobacterial membranes containing the pfam 02608 domain. YufN is involved in the import of nutrients into cells or the release of toxic products into the surrounding medium and functions at the expense of ATP hydrolysis (Locher et al., 2002). This class of proteins is the most important family of membrane transporters in *Halobacterium* NRC-1 genome. The other gene products identified by proteomic analysis has been found ThiC, ThiD, FumC, ImD2, GapB, TpiA, and PurE, which belong to a category of proteins involved in general metabolic function (Bernhardt et al., 1997). In addition, four unannotated gene products viz, Vng1807H, Vng0683C, Vng1300H, and Vng6254 of undetermined function have also been identified.

Heavy metal stress

Plants respond to heavy metal toxicity in different ways (Qadir et al., 2004; Agehi et al., 2009). Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, formation of peptide metal-binding ligand PCs (Grill et al., 1985) and MTs (Cobbett et al., 2000) and the expression of more general stress-response mechanisms, such as ethylene and stress proteins. A large number of stress proteins have been found be induced by heavy metal stress with a molecular mass of 10,000–70,000 KDa (Delhaize et al., 1989) in plants. In case of proteomics of nodules under heavy metal stress, only Cd has been taken in consideration (Shevchenko et al., 1996) and thus there is a large gap from this point of view. The number of proteins identified by 2D analysis in root nodules under Cd stress has been found to be up regulated or down regulated. Several proteins related to that of SNF (symbiotic nitrogen fixation) such as *nif*D, *nif*K, *nif*H and nitrogen regulatory protein II (GlnB) and PIIA (PtsN) and urease accessory proteins have been found to be affected by different heavy metal stresses. Twenty four nodule proteins have been subjected to N-terminal amino acid sequencing under heavy metal stresses in *M. trancatula* which helps to combat the heavy metal stresses. The proteome pattern of nodules has also been compared with that of mock inoculated roots and isolated bacteriods cells. The past separation technique of N-terminal sequencing and matrix assisted laser desorption time of flight (MALDI-TOF MS) in combination with bioinformatics have been used to determine putative identity of many of the individual proteins present in root nodules, which allows a high through put of samples with rapid analysis time (Qureshi et al., 2007), MALDI-TOF MS analysis have not only provided the likely identity of over 100 *S. meleloti* proteins but also confirmed that about 10% of protein species analyzed were isoforms under and four forms of n*if*H have been identified among the proteins novel to the bacteroid (compared with bacteria). Membranes and other hydrophobic proteins have been found probably underrepresented in 2DE gels at the present time. Therefore nodulins located in the membrane such as nodulins 26 in *S. meliloti* have been likely to be identified under heavy metal stresses.

Applications of N_2-fixing nodule proteomics

The application of proteomics associated with N_2-fixing root nodules in modern biology is growing, to sustain the exciting challenges for the future projected harsh environmental conditions such as increasing pollution, global warming, nutrient imbalance, etc. Significantly, proteomics helps us to know about the proteins of root nodules which are involved in nitrogen metabolism, carbon metabolism, and in other cell division processes (Natera et al., 2000). Several bacterial proteins involved in plant-microbe interactions allow knowing the actual mechanism of symbiotic relationship of host and pathogens. Obtaining the data of protein sequencing, proteins playing key role in response to abiotic stress may be focused along with to understand the functions of identified proteins/genes. Identification of a number of nodule-specific proteins may serve as physiological markers of tissue-specific protein expression. Putative unique proteins may provide valuable insight into the specialized physiological function leaves. Tissue-specific SDS-PAGE/2-DE protein profiles will provide reference maps for future proteomic comparisons of wild-type, genetic mutants, biotically and abiotically challenged plants. To know if, where, when and at what level a messenger will be translated, and the corresponding protein will accumulate. To help in study of the posttranslational modifications (such as cutting of the signal peptide, phosphorylation, glycosylation, etc). It is clear that the proteomic strategy has been responsible for the discovery of numerous new proteins, for cataloguing previously unknown cellular and sub cellular protein compliments (Christof et al., 2006). Proteomic data will enable to investigate the proteins/genes for better crop improvements in a better and effective manner.

Concluding Remarks

The importance of proteomics of N_2-fixing legume nodules in the post genomic era has been increasing day by day to sustain the various challenges. Proteomics greatly helps in identification of stress-responsive protein and understand SNF (symbiotic nitrogen fixation) under stress. A novel strategy of proteins for the development of plants tolerant to abiotic stress, pathogens and disease resistant plants can be improved. It can enable us to understand the actual symbiotic relationship between host and pathogens. Different putative marker genes and proteins can be identified for the development of transgenic plants. Identification of symbiosome proteins and their encoding genes will definitely provide more but important options for development of plants with better with standing potential under stress and more N_2-fixing efficient. In addition to this, post translational modifications, phosphorylation, signal peptides and glycosylation etc; may also be studied.

References

Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 13: 198–207.

Aghaei K, Ehsanpour AA, Shah AH, Komatsu S (2009) Proteome analysis of soybean hypocotyl and root under salt stress. Amino Acids 36: 91-98.

Allen EK (1958) Biological aspects of symbiotic nitrogen fixation, p. 48-118. In W. Ruhland [ed.], Handbuch der Pflanzenphysiologie, vol. 8. Springer-Verlag, Berlin.

Amiour N, Recorbet G, Robert F, Gianinazzi S, Dumas-Gaudot E (2006) Mutations in DM13 and SUNN modify the aspersorium responsive root proteome in arbuscular mycorrhiza. Mole Plant-Microbe Inter. 19: 988-97.

Arai Y, Hayashi M, Nishimura M (2008) Proteomic analysis of highly purified peroxisomes from etiolated soybean cotyledons. Plant Cell Physiol. 49: 526-539.

Beijerinck AMW (1888) Die Bacterien der Papition lionaceen-Knollchen Botan. Ztg. 46: 726-735.

Benabdellah K, Azcon-Aguilar C, Ferrol N (2000) Alterations in the plasma membrane polypeptide pattern of tomato roots (*Lycopersicon esculentum*) during the development of arbuscular mycorrhiza. J Exp Bot. 51: 747-54.

Beringer JE, Brewin NJ and Johnson AWB (1980) Effect of combined nitrogen on nodulation and growth of *Phaseolus vulgaris*. Expt Agric. 16: 303-311.

Bernhardt J, Volker U, Volker A, Antelmann H, Schmid R, Mach H, Hecker M (1997) Specific and general stress proteins in Bacillus subtilis- a two-dimensional protein electrophoresis study. Microbiology 143: 999-1017.

Bestel-Corre G, Dumas-Gaudot E, Poinsot V (2002) Proteome analysis and identification of symbiosis-related proteins from *Medicago trancatula* by two-dimensional gel electrophoresis and mass Spectrometry. Electrophoresis 23: 122-137.

Bestel-Corre G, Dumas-Gaudot E, Gianinazzi S (2004) Proteomics as a tool to monitor plant microbe endosymbiosomes in the rhizosphere. Mycorrhiza 14: 1-10.

Castillejo MA, Amiour N, Dumas-Gaudot E, Rubiales D, Jorrın JV (2004) A proteomic approach to studying plant response to crenate broomrape (*Orobanche crenata*) in pea (*Pisum sativum*). Phytochemistry 65: 1817-28.

Ceser AI, Gonzalez EM, Marino D, Landrea R, Larrainzar E, Quintana EG (2009) Physiological response of legume nodules to drought. Plant Stress 1: 1-31.

Chen HC, Higgins J, Oresnik IJ (2000) Proteome analysis demonstrates complex replicon and luteion interactions in Psyma-cured derivatives of *Sinorhizobium meliloti* strain Electrophoresis 16: 438-443.

Chen S, Harmon AC (2005) Advances in Plant Proteomics. Proteomics 6: 5504-16.

Christof R, Muralli S (2006) The application of proteomics to plant biology: a review. Can J Bot. 84: 883-892.

Cobbett CS, Goldsbrough P (2000) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Ann. Rev. Plant Biol. 53: 159–82.

Colditz F, Nyamsuren O, Niehaus K, Eubel H, Braun HP, Krajinski F (2004) Proteomic approach: identification of Medicago trancatula proteins induced in roots after infection with the pathogenic oomycetes *Aphanomyces euteiches*. Plant Mol Biol. 55: 109-120.

Dangeard PA (1926) Recherches sur les tubercles riadicaux des legumineuses. Botaniste 16: 1-275.

Delhaize E, Jackson PJ, Lujan LD, Robinson NJ (1989) Poly (glutamylcysteinyl) glycine synthesis in *Datura innoxia* and binding with cadmium. Plant Physiol. 89: 700–6.

Djordjevic MA (2004) *Sinorhizobium meliloti* metabolism in the root nodule: A proteomic perspective. Proteomics 4: 1859-72.

Djordjevic MA, Oakes M, Li DX, Hwang CH, Hocart CH, Gresshoff PM (2007) The Glycine max xylem sap and apoplast proteome. J. Proteome Res. 6: 3771-3779.

El-Sheikh EAE, Wood M (1995) Nodulation and N_2-fixiation by soybean inoculated with salt-tolerant rhizobia or salt-sensitive Bradyrhizobium in saline soil. Soil Biol. Biochem. 27: 367-661.

Estı´baliz L, Stefanie W, Wolfram W, Rube´n L, Cesar A, Esther MG (2007) *Medicago truncatula* Root Nodule Proteome Analysis Reveals Differential Plant and Bacteroid Responses to Drought Stress. Plant Physiol. 144: 1495-1507.

Ferguson BJ, Mathesius U (2003) Signaling interactions during nodule development. J plant growth Regul. 22: 47-72.

Fester TMK, Strack D (2003) A mycorrhiza-responsive protein in wheat roots. Mycorrhiza 12: 219-22.

Frank C, Nyamsuren O, Niehaus K, Eubel H, Braun HP and Krajinski F (2004) Proteomic approach: identification of *Medicago trancatula* proteins induced in roots after infection with the pathogenic oomycetes Aphanomyces euteiches. Plant Mol Biol. 55: 109-120.

Godovac-Zimmermann J, Kleiner O, Brown LR and Drukier AK (2005) Perspectives in spicing up proteomics with splicing. Proteomics 5: 699–709.

Grill E, Winnacker EL and Zenk MH (1985) Phytochelatins: the principle heavy-metal-complexing peptides of plants. Science 230: 574–6.

Hazelwood JL, Millar AH (2003) Integrated Plant Proteomics-Putting the green genomes to work. Funct Plant Biol. 2: 320-326.

Hellriegel H and Wilfarth H (1888) Untersuchungen Über die stickstoffnährung der Gramineen und Leguminosen. Beil. Ztscher. Ver. Dt. ZuckInd.

Jorge I, Navarro R M, Lenz C, Ariza S, Jorrin J (2006) Variation in the holm oak leaf proteome at different plant developmental stages, between provenances and in response to drought stress. Proteomics 6: 207–14.

Kav NNV, Srivastava S, Yajima W, Nidhi S (2007) Application of proteomics to investigate Plant microbe-Interactions. Current Proteomics 4: 28-43.

Kersten B, Burkle L, Kuhn EJ, Giavalisco P, Konthur Z, Lueking A, Walter G, Eickhoff H, Schnneider U (2002) Large-scale plant Proteomics. Plant Mol. Biol. 48: 133-141.

Krishnan HB, Natarajan SS, Mahmoud AA, Nelson RL (2007) Identification of glycinin and beta-glycine subunits that contribute to the increased protein content of high-protein soybean lines. J. Agric. Food Chem. 55: 1839-1845.

Kruger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L and Jones JDG (2002) A tomato cysteine protease required for Cf-2 dependent disease resistance and suppression of autonecrosis. Science 296: 744-747.

Lechtova-Trnka AI (1931) Etudes sur les bacte'ries des l6 legumineuses observations sur quelque chamiipignons parasites des nodosites. Botaniste 23: 301-530.

Lewis S, Handy RD, Cordi B, Billinghurst Z, Depledge MH (1999) Stress proteins (HSPs): Methods of detection and their uses environmental biomarkers. Ecotoxicology 8: 351–68.

Lin D, Tabb DL, Yates IJR (2003) Large-scale protein identification using mass spectrometry. Biochem. Biophys. Acta. 1646: 1–10.

Locher KP, Lee AT, Rees DC (2002) The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. Science 296: 1091-1098.

Loh J, Stacey G (2003) Nodulation gene regulation in *Bradyrhizobium japonicum:* a unique integration of global regulatory circuits. Appl. Environ. Microbiology 69: 10-17.

Martin EF (1999) Organismal, Ecological, and Evolutionary Aspects of Heat-Shock Proteins and stress Response: Established Conclusions and Unresolved Issues Amer Zool. 39: 857-864.

Mathesius U, Keijzers G, Natera SHA, Weinman JJ, Djordjevic MA, Rolfe BG (2001) Establishment of a root proteome reference map for the model legume *Medicago trancatula* using the expressed sequence tag database for peptide mass fingerprinting. Proteomics 1: 1424-1440.

McCoy E (1932) Infection by Bact. radiciola in relation to the microchemistry of the host's cell walls. Proc Roy Soc (London) Ser. B 110: 514-533.

Mesfin T, Samac DA,Vance CP (2005) Insights into symbiotic nitrogen fixation in *Medicago trancatula.* Mol. Plant-Microbe Inter. 19: 330-341.

Mithofer A, Muller B, Wanner G, Eichaker LA (2002) Identification of defense related cell wall proteins in *Phytophthora sojae* infected soybean roots by ESI-MS/MS. Mol. Plant Pathol. 3: 163-166.

Munchbach M, Dainese P, Staudenmann W, Narberhaus F, James P (1999) Proteome analysis of heat shock expression in *Bradyrhizobium japonicum*. Eur. J. Biochem. 263: 39–48.

Natarajan SS, Xu C, Bae H, Bailey BA, Cregan P, Caperna TJ, Garrett WM, Luthria D (2007) Proteomic and genetic analysis of glycine subunits of sixteen soybean genotypes. Plant Physiol. Biochem. 45: 436-444.

Natarajan SS, Xu C, Bae H, Caperna TJ, Garrett WM (2006) Characterization of storage proteins in wild (Glycine soja) and cultivated (*Glycine max*) soybean seeds using proteomic analysis. J Agric Food Chem. 54: 3114-3120.

Natera SHA, Guerreiro N, Djordjevic NA (2000) Proteome analysis of differentially displayed proteins as a tool for the investigation of symbiosis. Mol Plant Microbe Interact. 13: 995-1009.

Nutman PS (1956) The influence of the legume in root-nodule symbiosis: A comparative study of host determinants and functions. Biol Rev. Cambridge Phil. Soc. 31: 109-151.

Nutman PS (1958) The physiology of nodule formation, p. 87-106. In E.G. Hallsworth [ed.], Nutrition of the legumes. Butterworths, London.

Qadir S, Qureshi MI, Javed S and Abdin MZ (2004) Genotypic variation in phytoremediation potential of *Brassica juncea* cultivars exposed to Cd stress. Plant Sci. 167: 1171–81.

Qureshi MI, Muneer S, Bashir H, Ahmad J, Iqbal MI (2010) Nodule physiology and proteomics of stressed legumes. Adv Bot Res. 35: 1-47.

Qureshi MI, Qadir S, Zolla L (2007) Proteomic based dissection of stress responsive path ways in plants. J Plant Physiol. 164: 1239-1260.

Rolfe BG, Mathesius U, Djordjevic MA (2003) Proteomic analysis of legume-microbe interactions. Comp. Funct. Genome. 4: 225-228.

Rossignol M (2001) Analysis of Plant Proteome. Curr Opin Biotech. 12:131-134.

Sarma AD and Emerich DW (2006) A comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. Proteomics 6: 3008–3028.

Sarry J-E, Kuhn L, Lay PL, Garin J, Bourguignon J (2006) Dynamics of *Arabidopsis thaliana* soluble proteome in response to different nutrient culture conditions. Electrophoresis 27: 495–507.

Schaede R (1941) Untersuchungen and den Wurzelknollchen von *Vicia faba* und *Pisum sativum*. Beitr Biol Pflanz. 27: 165-188.

Selao TT, Nordlund S, Noren A (2008) Comparative Proteomic studies in *Rhodospirillum ruburm* Grown under Different Nitrogen conditions. J Proteome. 7: 3267-3275.

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Analyt Chem 68:850-858.

Shi F, Yamamoto R, Shimamura S, Hiraga S, Nakayama N, Nakamura T, Yukawa K, Hachinohe M, Matsumoto H, Komatsu S (2008) Cytosolic ascorbate peroxidase 2 (cAPX 2) is involved in the soybean response to flooding. Phytochemistry 69: 1295-1303.

Siria HA Natera, Guerreiro N, Djordjevic MA (2000) Proteome analysis of differentially displayed proteins as a tool for the investigation of symbiosis. Plant Microbe Interact. 13: 995-1009.

Sule A, Vanrobaeys F, Hajo´s GY, Van Beeumen J, Devreese B (2004) Proteomic analysis of small heat shock protein isoforms in barley shoots. Phytochemistry 65: 1853–63.

Thiellement H, Bahrman N, Damerval C, Plomion C, Rossignol M, Santoni V, de Vienne D, Zivy M (1999) Proteomics for genetic and physiological studies in Plants. Electrophoresis 20: 2013-2026.

Thorton HG (1930) The influence of the host plant in inducing parasitism in lucerne and clover nodules. Proceedings of the Royal Society of London B, Biological Sciences 106: 110–122.

Trevaskis B, Colebatch G, Desbrosses G, Wanrey M, Wienkoop S, Saalbach G, Udvardi M (2002) Differentiation of plants cells during symbiotic nitrogen fixation. Comp. Funct. Genomics 3: 151-157.

Vallorani L, Bernardini F, Sacconi C, Pierleoni R, Pieretti B, Piccoli G, Buffalini M, Stocchi V (2000) Identification of Tuber borchii mycelium proteins separated by two dimensional polyacrylamide gel electrophoresis using amino acid analysis and sequence tagging. Electrophoresis 21: 3710-6.

Van Wijk K (2001) Challenges and Prospects of plant Proteomics. Plant Physiol. 126: 501-508.

Veeranagamallaiah G, Jyothsnakumari G, Thippeswamy M, Chandra Obul Reddy P, Surabhi G-K, Sriranganayakulu G, Mahesh Y, Rajasekhar, Madhurarekha CH, Sudhakar C (2008). Proteomic analysis of salt stress response in foxtail millet (*Setaria italic* L. CV.) seedlings. Plant Science 175: 631-641.

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9: 244–52.

Ward HM (1887) On the rubercular swellings on the roots of *Vicia faba*. Phil. Trans. Roy. Soc. London, Ser. B 178: 539-562.

Willey JM, Sherwood LM, Woolverton CJ (2008) A Book on Microbiology.

Xia Y, Suzuki H, Borevitz J, Guo Z, Patel K, Dixon RA, Lamb C (2004) An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. EMBO (Eur. Mol. Biol. Organ.) J 23: 980-988.

Xu C, Sullivan JH, Garrett WM, Caperna TJ, Natarajan S (2008) Impact of solar ultraviolet-B on the proteome in soybean lines differing in flavonoid contents. Phytochemistry 69: 38-48.

Yeh KC, Peck MC, Long SR (2002) Luteolin and GroESL modulation in vitro activity of NodD. J Bacteriol. 84: 525-530.