ORIGINAL RESEARCH PAPER
Volume - 13 | Issue - 11 | November - 2024 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

PROTEOMIC ANALYSIS OF BACTERIAL CONSORTIUM TREATED CROP OF *VIGNA RADIATA* - LEAF AND SEED PROTEOME

Agricultural Science

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ABSTRACT

Interaction between bacteria and plant define multiple enzymes and chemical mechanism, elucidation of these mechanisms might be correlated with protein expression. Extraction and identification of proteins from the plant tissues was achieved with the help of (2-DE) two dimensional gel electrophoresis and mass spectroscopy. In this study we focused to identify the differentially expressed proteins by the treatment of microbial consortium. Most of the researchers have been conducted proteomic experiment in mung bean due to it is one of the important nitrogen fixer. Overall 28 proteins has been differentially, up regulated. Identified proteins were plays the role in the plant growth, plant hormonal, nutrient transport and stress related functioning. Proteins were expressed in the seeds of Vigna radiata than the leaves tissue.

KEYWORDS

Proteomic, *Vigna radiata, 2-DE gel electrophoresis, Plant Growth Promoting Bacteria* (PGPR)

INTRODUCTION

Proteomic tool plays important role to identify the functional and metabolic characteristic of plants, area including molecular, proteomics, 2-DE, MS with bioinformatics analysis provided the mechanism and protein expression by the plant microbe interaction (Cánovas *et al.*, 2004). With the help of bioinformatics Muneer et al., 2014 identified some of the proteins, which is responsible for stress and abiotic stress tolerance, henceforth they concluded that the finding of genes and proteins in the leguminous crop might provide the major mechanism of plants including the process of nitrogen fixation.

Proteomics study helps to separate the protein by 2D-PAGE with micro analytical technique like mass spectrometry and further it provide the information of genome sequence as well as protein profile (Mehta *et al.*, 2008). PGPR produced molecules like, simpler amino acid to larger proteins and polysaccharide in the rhizosphere (Rosier, Medeiros and Bais, 2018).

In human nutrition the seeds proteins plays a major role major role (Lam *et al.*, 1996), the proteins like phenylalanine, tryptophan involved in metabolic process and phytohomonal production and however the effect PGPR like *Azosopirillum* and *Azotobacter* significantly improved by amino acid and crude protein in seeds (Nosheen *et al.*, 2016). Mainly PGPR involves in the aminoacid synthesis such as lysine, glutamic acid, valine, serine, leucine and isoleucine (Babalola, 2010). In the leaves sample the protein changes was observed after the bacterial inoculation, and it promote the immune system to the crop (Lewis *et al.*, 2015).

Hashiguchi et al., 2017 reported to be in the analysis outcome of proteomic and metabolomic was undertaken from four different mung bean cultivars of China, Myanmar and Thailand. There were 210, 449 of metabolic compounds and proteins were spotted out in the seed coat, as well as 217 metabolic compounds and 480 proteins were identified from the seed flesh.

The *P. fluorescens* microbial inoculum modulate the seed composition and it improves the starch content of the seed, simultaneously the proteins which are water soluble and zeins are highly reduced by the application of these bacterial inoculation, *P. fluorescens* Pf4 alone or co inoculated with AM fungi in maize provided root elongation and increased leaves counts. (Berta *et al.*, 2014), Moreover the strain *Bacillus* sp Da_ 4 improved shoot length and oxidize the H2DCFDA. Proteins which are differentially expressed by the action of *Bacillus* sp Da_ 4 and *Bacillus* sp Oa_ 4 highly responsible for the defence system and cell development. These aspects point out the mutualistic interaction between the plants and bacterial strains, Tamošiūnė et al., 2018 identified variance in the protein expression by the effect of co cultivation of *Bacillus* spp.

MATERIALS AND METHODS

Field Experiment And Treatments

The preliminary field study was carried in the Vellore Institute Technology, Vellore, Tamil nadu with six experimental treatments (T1 - Panchagavya, T2 - Inorganic fertilizer, T3 - Microbial consortium, T4 - *Rhizobium* fertilizer, T5 – Manure, T6 – Control). Higher grain yield and nitrogen fixation was obtained in the treatment of microbial consortium (Indrapriyadharshini and Karthikeyan, 2020). Based on the previous study results, bacterial consortium treated and control plant tissues (Seed and leaf) were taken for the analysis of proteome.

Protein Extraction From Green Gram Seed

The green gram seeds were finely grounded by using liquid nitrogen, the grounded samples (200 mg) ware taken and resuspended in the 2ml of 10% TCA suspended acetone with 0.07% of DTT (Dithiothreitol). The Samples were kept for 1 hour at -20˚C for protein precipitation. The precipitated proteins were separated out by centrifugation (15 min at 14000rpm). Protein pellet was washed five more times by using acetone containing 0.07% of DTT. The pellet was completely allowed to air dry (not more than15 min in the RT). The final pellet was dissolved in rehydration buffer containing (8M urea, 2% CHAPS, 0.7% DTT, 0.5% ampholytes (3-10)). The buffer was kept at 4˚C for overnight dissolving the pellet; the crude proteins and insoluble molecules were removed by centrifugation (14000 rpm for 10 min at 4˚C).

Protein Extraction From Leaves

The leaves were rinsed with sterile distilled water to remove all the soil particles, the leaves were pulverized by using liquid nitrogen in the pre chilled pestle and mortar. The grounded leaves tissues (250 mg) were dissolved in 10% TCA in acetone with 0.07% of DTT. The extract was sonicated in ice by using ultrasonic bath sonicator (PCI analytics ultrasonics) for 20 min. The extract was incubated at -20˚C for 1 hour. After protein precipitation the pellet was obtained by centrifugation at 14,000 rpm 30 min. The collected pellet was washed around five more times with acetone containing DTT (0.07%), The final pellet was air dried at room temperature for 10 min, Not more than 10 min and it is resuspended in 500 µl of rehydration buffer (8M urea, 2% CHAPS, 0.7% DTT, 0.5% ampholytes (3-10)) for overnight 4˚C. The crude protein was obtained from the rehydration buffer after centrifugation at 14000 rpm for 10min and it was stored at -80˚C.

Protein Quantification

The solubilized proteins were obtained from the centrifugation. Proteins from the seeds and leaves were quantified from the collected

pellet by Bradford method (Bradford, 1976).

Two Dimensional Polyacrylamide Gel Electrophoresis (2-DE PAGE)

After quantification 100 µg (leaf), 150 µg (seed) of proteins were used for 2DE gel electrophoresis. The protein samples were loaded (250 µl) in the immobilized pH gradient IPG gel strips (pH 4-7). The gel was kept for 12 hours rehydration. After complete rehydration the isoelectric focusing was carried out at 20˚C. 250 V for 15min to eliminate the charge contaminants and salt ions , 4000 V for 2 hours, 4000 V for 20000 Vh and 500 V 30 min gradually. After isoelectric focusing the IPG strip was removed from the focusing tray. Before second dimension the strip was equilibrated by using equilibration buffer (6M urea,75mM Tris HCl, pH 8.8, glycerol 29.3%, SDS 2%), First equilibration was done by using 1% of DTT in equilibration buffer, followed that 4% of iodoacetamide was used for the alkalization. 15% of SDS (sodium dodecyl sulphate) gel was performed after completing the equilibration steps. The 2-DE gel was allowed for the staining for overnight in (R-250) coomassie brilliant blue. After destining the spot was identified and compared. The selected spots were subjected to MALDI-MS/MS analysis.

RESULTS

The proteomic analysis revealed that expression of more number of proteins with or without treatment, and the proteins which is noticed in this experiment was first reports in the *Vigna radiata* crop. Hence the identified proteins may help to further analysis on the same field. The effect of microbial consortium on *Vigna radiata* protein expression was investigated by 2DE gel electrophoresis. To identify the molecular mechanism between microbial consortiums treated crops with control. This research study helps to point out differentially expression of the protein during the application of microbial consortia.

Leaf and seed protein was extracted by TCA acetone method with slight modification from the previous reports. The TCA acetone method was done with ultra-sonication and it was used for 2DE analysis. The extracted protein was solubilized by rehydration buffer; concentration of protein was major consideration to process the 2DE, hence the protein concentration was obtained for the entire samples by standard Bradford method. The sample concentration was identified with Bovine serum albumin (BSA) standard, control and treatment samples concentration was adjusted to avoid dissimilarity. The unknown protein size was identified with protein ladder in the range of 10 to 100 kDa and the range of p*I* is from 4-7 was identified by coomassie brilliant blue staining techniques.

The protein spots were evaluated from the 2DE gel image by using Imagemaster-2D (Fig 1). Total 514 hundreds of protein spots were detected, they were total 132 Spots has been noticed in leaf protein, in addition 382 spots from seed sample. Rather than leaf protein the seed protein has been expressed highly. The protein expression between control and treated sample, the high protein intensity was observed in the treated sample of both part (leaf and seed) of the plant tissue. Moreover the protein up regulation of has been viewed more number in the treated sample. The experimental report of seed proteome revealed that the storage protein was abundance and as well as during the process of glycolysis and TCA metabolism the seed protein expression seems to be decreased (Kaz *et al.*, 2013).

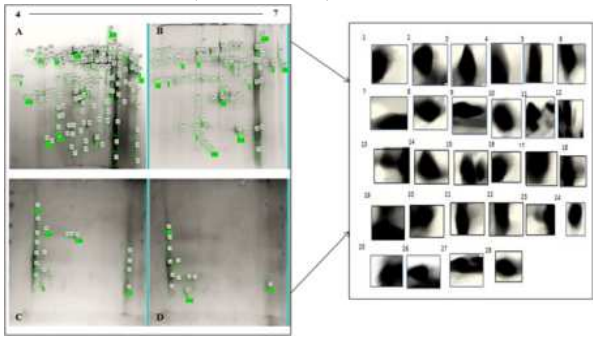

Figure 1: TCA acetone extracted seed and leaf protein was separated by 2DE (A) Microbial treatment seed proteins (B) Control seed treatment (C) Microbial treatment leaf proteins (D) Control leaf proteins

Totally twenty eight protein spots (Spot number 1-28) were highly up-regulated and differentially expressed under the treatment of microbial consortium (treatment of *B. subtilis, P. fluorescens* and *L. plantarum*). Moreover remaining noticed spots were observed commonly both treated and control tissues.

Differentially Regulated Proteins In Leaf And Pod Tissue By 2DE

Effect of microbial consortium on *Vigna radiata* protein expression was investigated by 2DE gel electrophoresis. To identify the molecular mechanism between microbial consortiums treated crops with control. This research study helps to point out differentially expression of the protein during the application of microbial consortia.

Leaf proteome analysis was detected and high scored proteins were only selected for classification. The interaction between plant and microbes expressed two hundred proteins, further the important function of the protein has been identified by the UniProt.

In overall leaf sample proteome revealed total seven spot which common for both the treated and control within that the mascot identified proteins **(Table 1)** such as protein probable nucleolar protein 5-1 and probable mixed-linked glucan synthase (Spot ID 5) has been identified both control and treated leaf sample of *Vigna radiata*. Probable mixed-linked glucan synthase 7 (Spot ID 8) was noticed 2.56 fold increased in the treated compared than control and protein probable nucleolar protein 5-1 was 4.90 fold increased in the treated samples. Henceforth microbial treatment highly increase the various phosphorylation activity and as well as it plays important role in the synthesis beta-D-glucans. Comparatively leaf protein, seed protein spot was observed maximum level with high intensity **(Table 1)**.

In overall experiment the plant growth associated protein was noticed upregulated such as Kinesin –like protein KIN-UA (Spot ID 12), Canavalin (Spot ID 13), Homeobox-leucine zipper protein HDG1(Spot ID 14), 1-Cys peroxiredoxin PER1 (Spot ID 16), Granule bound starch synthase 1b, chloroplastic/amyloplastic (Spot ID 19), Uricase-2 isozyme 1 (Spot 24). Some of the proteins were correlated with phytohormonal production like Auxin-responsive protein IAA23 (Spot 2), Type IV inositol polyphosphate 5 phosphatase 6 (Spot 6). Various functions of proteins were observed in the seed and leaf tissue of *Vigna radiata* (**Fig 2).**

Auxin-responsive protein IAA23 (Spot ID 2) proteins involved in the auxin regulation and metabolism, In many aspects the particular protein which regulates the plant growth, moreover this specific protein highly helpful for the formation of nodules from tryptophan by using Trp-dependent pathways.

Type IV inositol polyphosphate 5-phosphatase 6 (Spot ID 2) major contribute for the cell growth differentiation, development of cell structure like xylem and phloem. This protein highly involved in the Acetoin biosynthesis process and auxin signalling pathway.

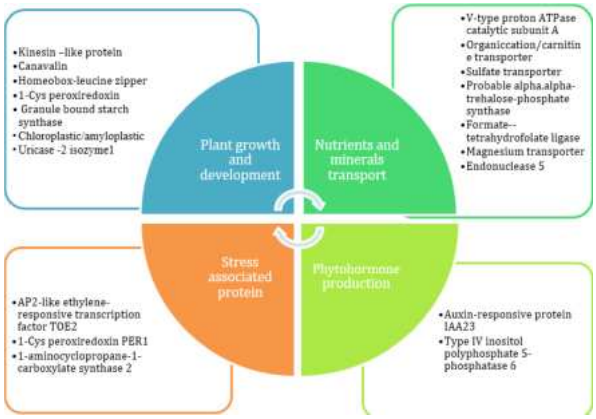

Figure 2. Various Functions Of Proteins Involved In Plant Metabolism And Growth

Panther analysis helps to classify the proteins abundance, it was done for the proteins which is highly abundant in the treatment of microbial consortium, based on the following classification, the MASCOT identified proteins were categorized. 1) Molecular function 2) Biological process. Within those classifications binding protein, catalytic activity and molecular transducer activity containing proteins

were monitored in the leaf and seed tissues of microbial treatment processed plants.

Table 1.1 Differentially regulated proteins in leaf and pod tissue by 2DE

Spot ID	Leaf & seed from microbial treatment	Accession number	Gene	Homology	Mascot Score	Biological function	Identified plant species
1	Leaf	Q9SBA5	ITPK1	Inositol-tetrakisphosphate 1-kinase 1	75	Involved in the inositol phosphorylation	*Arabidopsis thaliana*
2	Leaf	Q69VE0	IAA23	Auxin-responsive protein IAA23	33	Auxin response gene repressor	*Oryza sativa subsp. japonica*
3	Leaf	Q8H1Q1	TL20.3	Thylakoid lumenal protein TL20.3, chloroplastic	80	Unknown function	*Arabidopsis thaliana*
4	Leaf	P27163	CAM72	Calmodulin- 2	56	Mediate calcium and metal binding	*Petunia hybrida (Petunia)*
5	Leaf	O04658	NOP5-1	Probable nucleolar protein 5-1	95	Involved in the biogenesis of 16 S ribosome	*Arabidopsis thaliana*
6	Leaf	Q9LR47	IP5P6	Type IV inositol polyphosphate 5-phosphatase 6	50	Involved in the abscisic acid-activated signaling pathway	*Arabidopsis thaliana*
7	Leaf	Q9SLG8	SSl2	Protein Strictosidine synthase-like 2	50	Involved in biosynthetic process	*Arabidopsis thaliana*
8	Leaf	Q94GM9	CSLF7	Probable mixed-linked glucan synthase 7	60	In plant cell wall biogenesis	*Oryza sativa subsp. japonica*
9	Leaf	Q9LVG2	TOE2	AP2-like ethylene-responsive transcription factor TOE2	47	Ethylene signaling pathway	*Arabidopsis thaliana*
10	Leaf	Q9XFB7	NAS9	Nicotianamine synthase 9	46	Synthesizes nicotianamine	*Hordeum vulgare*
11	Seed	Q9SA14	IMDH3	3-isopropylmalate dehydrogenase 3, chloroplastic	173	Synthesizes of nicotianamine	*Arabidopsis thaliana*
12	Seed	Q9FZ06	KINUA	Kinesin-like protein KIN-UA	79	ATPase activity, Root development	*Arabidopsis thaliana*
13	Seed	P50477	Nill	Canavalin	69	Seed storage protein,	*Canavalia ensiformis*
14	Seed	Q9M2E8	HDG1	Homeobox-leucine zipper protein HDG1	62	Development of floral organ	*Arabidopsis thaliana*
15	Seed	Q84LK3	BADH2	Betaine aldehyde dehydrogenase 2	54	Dehydrogenase	*Oryza sativa subsp. japonica*
16	Seed	A2SZW8	PER1	1-Cys peroxiredoxin PER1	100	Helps for plant germination during the stress	*Zea mays*
17	Seed	Q9T010	At4g11040	Probable protein phosphatase 2C 54	78	ion transporter	*Arabidopsis thaliana*
18	Seed	Q2QM62	KIN14H	Kinesin-like protein KIN-14H	64	Motor protein	*Oryza sativa subsp. japonica*
19	Seed	Q8LL05	Nill	Granule bound starch synthase 1b, chloroplastic/ amyloplastic	54	Synthesis of amylose in endosperm	*Hordeum vulgare*
20	Seed	Q39291	CVA69.24	V-type proton ATPase catalytic subunit A	52	ATP binding and proton transport	*Brassica napus (Rape)*
21	Seed	O64515	OCT2	Organic cation/carnitine transporter 2	55	ATP binding and ion transport	*Arabidopsis thaliana*
22	Seed	P18485	ACS2	1-aminocyclopropane-1-carboxylate synthase 2	51	Involved in ethylene synthesis	*Solanum lycopersicum*
23	Seed	Q9FEP7	SULTR1;3	Sulfate transporter 1.3	49	Involved in sulfate assimilation	*Arabidopsis thaliana*
24	Seed	P04670	Nil	Uricase-2 isozyme 1	134	Formation of nodulation	*Glycine max*
25	Seed	Q9ZV48	TPS11	Probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 11	84	Transferase activity	*Arabidopsis thaliana*
26	Seed	P28723	Nill	Formate--tetrahydrofolate ligase	81	Ligase and one carbon metabolism	*Spinacia oleracea*
27	Seed	B8APK3	MRS2-A	Magnesium transporter MRS2-A, chloroplastic	58	Magnesium and ion transporter	*Oryza sativa subsp. Indica*
28	Seed	F4JJL3	ENDO5	Endonuclease 5	88	Metal and ion binding	*Arabidopsis thaliana*

Comparatively the molecular and biological functions between seeds and leaves were differentially expressed. More level of protein expression was identified in the seed tissue of mung bean compered the leaves. Hence the seeds proteome analysis comprises of more number proteins, same way some of the seed storage proteins were noticed (Spot ID 13) in the MASCOT results.

Our previous field experiment study report, correlated with this proteomic analysis, microbial consortium applied crops expressed more number nodules, similarly the abundance of legume inducing protein were higher in the treatment of microbial consortium, not in the control (Spot ID 24).

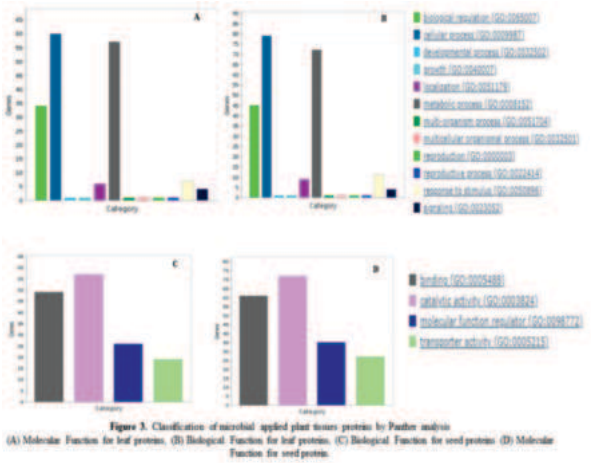

Figure 3. Classification of microbial applied plant tissues proteins by Panther analysis (A) Molecular Function for leaf proteins, (B) Biological Function for leaf proteins, (C) Biological Function for seed proteins (D) Molecular Function for seed protein

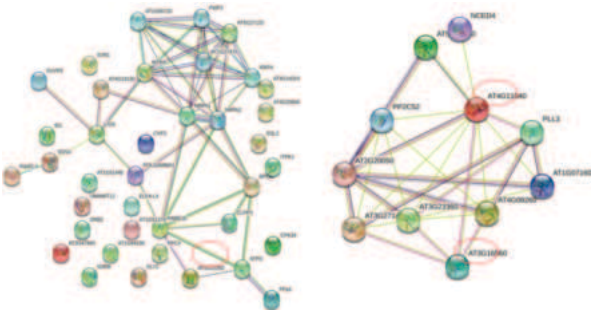

Figure 4: String data base revealed the interaction of proteins with various proteins

AT3G16560 genes which are involved in the magnesium ion transport and which are identified in around 23 plants. AT3G16560 genes interacted with major ten genes. Moreover AT3G16560 gene interact with AT4G11040 (Probable protein phosphatase 2C 54), it was highly increased by the application of microbial consortium. AT1G12250 (Thylakoid lumenal protein TL20.3, chloroplastic(Spot Id 3)) there is no clear evidence for its function but this particular protein interacted with multiple proteins and those genes where involved in the ATP binding and ion transport. The overall outcome of proteomics analysis strongly correlated with nutrient transport and cell growth development.

DISCUSSION

The aim of the study was to understand and identify the plant beneficial microbial consortium effects on mung bean protein expression. Identified protein were correlated with previous research study (Indrapriyadharshini and Karthikeyan, 2020)

There were genes like CVA69.24 and OCT2 from V-type proton ATPase catalytic subunit A and organic cation/carnititine transporter 2 involved in metabolic and energy transports. CVA69.24 which is reported for the increase seed elongation and initiation of seed trichomes (Smart *et al.*, 1998). OCT2 gene used to deliver the drug in the medical field; hence these two genes were expressed in the plant tissue, which will support the nutrients transports within the plant cell.

It was reported that Long chain acyl-coenzyme A synthatases (LACs) showed maximum level of activity with fatty acids, which plays important role in the structural formation and lipid storage in the tissues of Arabidopsis (Shockey, Fulda and Browse, 2002), further the same protein was noticed in the leaf of *Vigna radiata* without any treatment.

Uricase-2 isozyme 1 was upregulated by the activity microbial inoculum, moreover the Uricase II mostly produced on the free polysomes in the process of nodule development, it was reported in the crop of *Glycine max*. Nguyen et al. 1985 reported that they are two numbers of uricase activity has been identified in the stage of root development; one was in the earlier seedling and another in the root nodules. Moreover the enzyme uricsae was observed in the *E. coli* cells of cytoplasmic fraction and it catalyzes the allantoin conversion from the uric acid, further the function was carried out in the uninfected nodules containing peroxisomes (Suzuki and Verma, 1991), hence our study report suggests that the inoculation of microbial cells influence the uricase II production in the seed of green gram.

In the seed proteome analysis Probable protein phosphatase 2C 54 was increased 1.73 fold by the microbial treatment similarly probable protein phosphatase 2C 54 (PP2C) (P2C54_ORYSJ) was reported in the crop of rice and Arabidopsis, it was highly observed in the stress responses and as well as the crop development. Protein phosphatase involved in the signaling pathways and some important regulatory pathways in rice (Xue *et al.*, 2008). PP2C act in the abscisic acid response and seed germination process (Yoshida *et al.*, 2006).

3-isopropylmalate dehydrogenase 1, chloroplastic was participated in the biosysnthesis of leucine and moreover it involves in the oxidative decarboxylation in the biosynthesis of glucosinolate (He *et al.*, 2011), In the present study, first time we have noticed the protein in the seed of *Vigna radiata* which is treated with microbial inoculants.

Protein Type IV inositol polyphosphate 5-phosphatase 6 helped for the nutrient transport and cell structure synthesis, this specific protein was upregulated in the leaf of mung bean which is treated with microbial consortium. Balanced level of phosphatidylinositol-4,5-biphosphate involved in the root cell structure formation, the imbalance proposition may affect the cell dimensions (Rodriguez-Villalon *et al.*, 2015).

This research study on mung bean proteins proved with various functioning aspects, all the identified proteins were simultaneously involved in the plant growth and nutrient transport. The listed proteins were highly expressed by the treatment of microbial consortium.

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