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# PROTEOMIC STUDY ON GROWTH PROMOTION OF PGPR INOCULATED AEROBIC RICE (*ORYZA SATIVA* L.) CULTIVAR MR219-9

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**Abstract**

The plant growth promoting rhizobacteria (PGPR) perform substantial growth enhancement of aerobic rice. Study was conducted at Universiti Putra Malaysia using 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) strategy to identify mechanisms for rice plant growth promotion by PGPR inoculation. In this study, diverse expressed proteins were determined by mass spectrometry (MS). Aerobic rice seedling (MR219-9) was grown in the soil and PGPR strains of *Stenotrophomonas maltophilla* and *Bacillus* spp. were inoculated separately and or as combined bacterial consortium. Leaf sheath and other plant parts were collected after 45 d of transplanting for the analysis of proteins. A total of 153 spots were found and from which 12 proteins were identified. All proteins were varied in MS analysis and exposed the differential expression. The identified proteins were tolerant to abiotic stresses (13.2%), disease resistance (10%), oxidation reduction process (10%), photosynthesis (16.62 μmol $CO_2$ $m^{-2}$ $s^{-1}$), involved in the protein synthesis (23.28%), metabolism (13.6%) and related to internal plant physiological functions (13.29%). Beside protein identification, phenotypic characters, such as plant height and photosynthetic activity were measured. The highest plant height and length of root, tiller numbers and nutrients uptake were observed in PGPR inoculated treatments. PGPR inoculation increased leaf chlorophyll contents and net photosynthesis rate of inoculated aerobic rice. Hence, using plant proteomic approach it is proved that various designated proteins are responsible for the plant growth promotion of PGPR inoculated aerobic rice cultivar MR219-9.

**Key words:** 2-D PAGE, Bacterial consortium, Rice leaf sheath, Plant growth promotion, Proteomics.

## Introduction

The rice (*Oryza sativa* L.) commonly grown under flooded conditions and utilizes up to 43% of the world's irrigation resources (Bouman *et al.,* 2007). Due to climatic change, water scarcity is raising main issue for agriculture in the coming years and about 15 to 20 million hectares of wetland rice will be affected to some grade of water scarcity (Tuong & Bouman, 2003). Under this situation, water-saving technologies like, rotate wetting and drying, non-flooded mulching cultivation, and aerobic rice cultivations are being emphasized for the enhancement of rice production (Belder *et al.,* 2007). Aerobic rice is one of the approaches for water saving without sacrificing yield and highly fertilizer responsive adaption of rice genotypes are cultivated without flooded water. Generally, water use for aerobic rice is 30–70% lower than the wetland rice and dependent on the irrigation water management practices (Bouman *et al.,* 2005). Therefore, a special nutrient and water stress management with PGPR may lead to yield improvement of aerobic rice cultivars (Cakmakci *et al*., 2006).

Since ancient time it is known that microorganisms perform a significant role in the agriculture, particularly to supply soil nutrients for the growth and development of crops, and to reduce the usage of inorganic fertilizers (*Cakmakci et al.,* 2006). Several bacterial strains such as free living $N_2$-fixing and phosphate solubilization association with the plants' rhizosphere are capable to enhance rice plants growth (Naher *et al.,* 2016; Panhwar *et al.,* 2012). Therefore, the application of bio-fertilizers or bio-control managers in crop development has recently drawn the attention of the several researchers. These type of bacteria have been termed as 'plant growth promoting rhizobacteria' (PGPR) and amongst them, bacterial strains from the genera *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcaligenes*, *Aarthrobacter*, *Acinetobacter* and *Flavobacterium* are important for enhancing plant growth These rhizobia have been recognized to be related in rice plant rhizosphere (Naher *et al.,* 2009) and are reported to produce phytohormones (e.g. IAA) and large amount of organic acids which are responsible for phosphate solubilization (Panhwar *et al.,* 2014; Panhwar *et al.,* 2012). Other beneficial activities to improve crop growth are: high germination rate, plant root growth, plant leaf area, leaf chlorophyll value, Mg, N and amount of protein, hydraulic-activity, drought resistance and salt stress, dry biomass and late leaf senescence (Naher *et al.,* 2011; Lucy *et al.,* 2004). It is well known that the performance of PGPR formulated bacterial consortium is better than that applied to the crops as individuals (Jorrı´n *et al.,* 2006).

Proteins are the main key agents to play the role of gene expression and straightly related for the metabolism and cellular developmental processes in the plant system. Proteomics is going to be an influential tool for the agriculture research, giving new knowledge by utilizing classical biochemical strategies in various biological methods, such as growth enhancement and reactions against to the different plant stresses (Hakeem *et al.,* 2012; Shoresh & Harman, 2008).

Nevertheless, a very less information is available on plant and PGPR interface to growth of plant and its promotion up to molecular level. A 2-D PAGE approach has been widely consumed to know response against the plant stress might be due to the less water or excessive nutrients. Furthermore, it allows to outcome the transduction pathways signals and the post translational changes of proteins that express different functions. Furthermore, earlier study has exposed that in *Trichoderma harzianum* and maize interface, protein in metabolic pathways is persuaded by *T. harizianum*. Furthermore, comprising interface among *Pseudomonas fluorescens* strain KH-1 and rice, it was proven that due to the bacterial activity it had promoted the rice crop (Saveetha, 2009). The 2-D PAGE determination of leaf sheaths taken from bacterial non-inoculated /inoculated plant treatments which were visible the commencement of few prominent proteins elaborated for the energy metabolism system in plant cells. Hence, current study was carried out to find the efficiency of PGPR in the aerobic rice growth promotion and to identify the 2-D PAGE protein profiling involving in order to understand its mechanism for growth and development process.

## Materials and Methods

**Experimental site:** The glasshouse study was conducted at the University of Putra Malaysia, Serdang, Malaysia (latitude of 30.0° N and longitude of 101.70° E). An Inceptisol (Serdang series) about 10 kg $pot^{-1}$ soil was taken and the aerobic rice cultivar (MR219-9) was selected for this study. The experimental treatments were: a) control, b) *Bacillus* sp. (Sb13), c) *Stenotrophomonas maltophilla* (Sb16), d) *Bacillus* sp. (Sb42), e) *Bacillus* sp. (PSB16) and f) consortium of all bacteria. Nitrogen (urea), full dose of phosphate fertilizers triple super phosphate (TSP) and murriate of potash (MOP) were given at equivalent of 120, 30 and 60 kg $ha^{-1}$, respectively in the control pot. Leaf chlorophyll content and photosynthesis data were taken at 45 day of inoculation. The shoot and root length and number of tillers were calculated after 45 days of the transplantation. Soil nutrient contents and plant tissue analysis were carried out after harvest.The study was conducted in a completely randomized design (CRD) consisting of four replicates.

**Biochemical properties of the plant growth promoting bacteria:** The plant growth promoting rhizobacteria used in this study were free living $N_2$-fixing bacteria Sb13 (*Bacillus* sp.), Sb16 (*Stenotrophomonas maltophilla*), Sb42 (*Bacillus* sp.) and phosphate-solubilizing bacteria, PSB16 (*Bacillus* sp.). The strains have the ability to produce phytohormones (e.g. IAA), organic acids and enzymes (phosphatase and phytase) as well as able to solubilize phosphates and fix atmospheric nitrogen (Nakkeeran *et al.,* 2005; Amin *et al.,* 2004). The biochemical properties of these strains are given in (Table 1).

**Rice seedlings inoculation and transplanting:** The familiar rice variety of Malaysia MR219-9was selected and rice seeds were carefully sanitized followed the method of Amin *et al.,* (2004). First of all seeds rice were grown on a tray using filter paper. The sterilized distilled water was given to moisturise rice seeds and seedlings were left there for seven days, while 3 rice plants were transferred in every pot. Inoculum was cultured in nutrient broth. After 3 days of growth, bacteria cells were washed using phosphate buffer (pH 6.8) solution and exactly $1 \times 10^9$ populations of each bacteria was applied according to the treatments. In the mixed inoculum treatment, all of the strains were mixed before washing cells and washed cells were applied to the plant maintaining same population.

**Analysis of soil and plant samples:** The soil pH was taken in soil water ratio 1: 2.5 using PHM-210 Standard pH meter (Benton, 2001). Soil total organic carbon was done by LECO CR-412 and total N by Kjeldahl digestion method (Bremner & Mulvaney, 1982). While soil available P was determined by Bray 2 method (Bray & Kurtz, 1945). The exchangeable cations such as Ca, Mg, and K were analysed by the method of Bremner & Mulvaney. (1982) using atomic absorption spectrophotometer (AAS) and exchangeable Al using 1 M KCl extracting method by AAS (Kotze *et al.,* 1984). At harvest, agronomic parameters and uptake of nutrients were calculated following the method of Dobermann & Fairhurst (2000). The total nitrogen from plant sample was analysed by Kjeldahl method (Bremner & Mulvaney, 1982) and total tissue P was estimated by wet digestion method (Havlin & Soltanpour, 1980) whereas, rest of nutrients were analysed by dry ashing (Ryan *et al.,* 2001). However, amount of protein present in rice grain were measured applying Jones's factor [Protein % = N × 5.95], adopted from Merrill & Watt (1973).

**2-D PAGE Analysis Sampling:** Rice leaf sheaths were taken in liquid nitrogen after 45 days of sowing and kept at -80°Cfor proteomic analysis. The research study was done in duplicate (biological replications) including several replications. However, for the protein extraction samples were separated from the various treatments and mixed and pooled protein samples were equally disseminated into 3 aliquots that attended as the sub-replications of the samples.

**Table 1. Bio-chemical properties of the applied PGPR.**

| Strains | Accession number | IAA ($mg\ l^{-1}$) | Production of organic acid ($mg\ l^{-1}$) | | | | BNF ($^{15}N$ study) | P solubilization from PR in soil ($^{32}P$ study) | Enzyme production |
|---|---|---|---|---|---|---|---|---|---|
| | | | OA | MA | SA | PA | | | |
| *Bacillus* sp. (strain Sb13) | JQ820254 | 38 | - | - | - | - | 61 kg N $ha^{-1}$ | - | Cellulase |
| *Stenotrophomonas maltophilla* (Sb16) | JQ820255 | 56 | - | - | - | - | 52 kg N $ha^{-1}$ | - | Cellulase |
| *Bacillus* sp. (Sb 42) | JQ820260 | 33 | - | - | - | - | -- | - | Cellulase and Pectinase |
| *Bacillus* sp. (PSB16) | JX103827 | 6.78 | 0.02 | 0.05 | 0.24 | 0.01 | +ve | 86% | Phosphatase and phytase |

IAA = Indoleacetic acid, OA = Oxalic acid, MA = Malic acid, PA = Propionic acid, BNF = Biological nitrogen fixation, PR = Phosphate rock and N = Nitrogen

**Protein extraction:** The frozen leaf sheath samples were meshed by mortar using liquid nitrogen and adjourned in 10% trichloracetic acid in acetone and dithiothreitol [DTT] 0.07% and preserved at 20°C for 1 h, then centrifuged for 15 min at 35000 rpm. After centrifuge, pellets were properly cleaned by ice cold acetone comprising DTT 0.07% at -20°C for 1 hour and about 15 min centrifuged at 35,000 rpm. Same process was repeated till supernatant to be cleared from the chlorophyll contents. At last pellet sample (precipitated) was lyophilized for 2 hrs. The dried powder was utilized for extraction dissolved in 350µL of lysis buffer adjusted at pH 6.8 (containing 7-M urea, thiourea 2 M, CHAPS 4%, ampholytes (Bio-Rad) 0.5% and DTT 0.7%). The protein extraction was done at 37°C with occasional vortex. Exact 1 h of incubation, cell debris was pelleted by the centrifuging for 30 min at 35,000 rpm at normal temperature. However, the supernatant was dispersed in 100 µL aliquots and retained at -80°C prior to 2-DPAGE analysis (Kim *et al.,* 2009). The protein was measured by 2-D Bradford kit (Bio-Rad-laboratories), Hercules, CA with BSA standard procedure.

**2-D PAGE:** The protein (about 100 µg of plant sample) including untreated and soil treated samples were isolated by 2-D PAGE. The first dimension, the immobilized pH gradient (IPG) strips with pH 4 to7 was utilized. Electrophoresis was done at 500 V for 1 h, trailed by 1000 V for 1 h and 2950 V for 24 h. Then protein was isolated by SDS-PAGE in 2nddimension by 12% polyacrylamide gels-(Salekdeh *et al.,* 1999). While gels were marked by silver staining method, each set of the gels ran with high resolution running at various times prior to further analysis. The similar several spots were enumerated by Melanie III (GeneBio-Geneva, Switzerland) and scanned with a densitometry.

**Gel image and-data analysis:** The 2-dimensional-(2D) gels observed through Bio-Rad GS-710 [Calibrated Imaging Densitometers and valuation of proteins forms], was executed by Image-Master™ PD-Quest software of Bio-Rad. The optimised parameters were; saliency-2.0, limited threshold 4 and minimum area 50. The amounts of proteins were standardized by landmark proteins using interior standardization. However, the protein spots associated with the total volume were measured and counted by the amount of a whole spot from the protein sample.

**The procedures for scoring-analysis:** The sample of the proteins were detected and calculated by the Gaussian's method. The cluster of the protein was clarified consequently through line and equivalent, respectively. PDQuest-automatically analyses to enumerate the standard in the sample size. However of similar spot, each protein during volume (%) was calculated as its amount dispersed by taking whole volume of the same spots and stated to hence forward by concentration. While to compare the ability of each polypeptide, PROC-GLM model was utilized with Statistical Package (1990);

$$Yijk = Vi\ 1Nj\ 1\ VNij\ 1\ Bk\ 1\ NBjk\ 1\ Eijk$$

whereas, Vi is the effect of variety, Nj is the effect of nitrogen, VNij is the line between the rice genotype and N rate, Bk is the replication or block influence, NBjk is the line for the treatments and block, and remaining effect denotes by Eijk. All properties were analysed in inconsistency of the remaining (Eijk) without the effect of treatments that was established by NBjk in relation to take with the divided plot design and only those were used for further analysis which were in considerable quantities with two times higher and having reproducible changes (thrice replicated).

**Protein digestion:** The prominent spots of protein samples with in preparative-gels were detached. Whereas observable protein amounts were reduced through Mass-Prep station;: Waters, Exotic-protein spots were de-coloured with volume of 50µL of ammonium carbonate (50 mM) and about 50µL of 50% of acetonitrile was cleaned once with 50µL of ammonium carbonate (100 mM) and dehydrated acetonitrile (50 µL). After that the protein digestion was continued with 6 µL of 1 trypsin and 25 µL of ammonium carbonate (50 mM) for 5 hrs at 37°C temperature. After digestion proteins were further separated twice in1% formic acid (30 µL) and 12 µL formic acid 1% / 50% of acetonitrile, respectively. Finally collected proteins were collectively conserved on a PCR plate with 4°C of temperature in connection with additional study.

Identification of protein and sequencing by 2-D Nano LC-MS/MS: The identification and sequencing of the proteins were implemented by2-imensional liquid chromatography ESI-MS-Agilent1100 series 2DnanoLC MS-and tryptic digested protein procedure was continued to track by the inverse phase of isolation method. The peptides were observed by electrospray ionization mass spectrometry with the Shimadzu Standing nano HPLC system joined to a 5600-Triple TOF mass-spectrometer. The tryptic-peptides were laden into Agilent Zorba × 300SB-C18, 3.5 µm using Agilent Technologies and separated with a linear slope of water/acetonitrile/0.1% formic acid (v/v). The spectra were determined to identify proteins followed by the Mascot-sequence-matching software with Ludwig NR-database.

**Database exploratory with spectra (MS/MS):** The spectra (MS/MS)were utilized to explore beside the NCBI non laid off protein database in MS/MS Ion Search Engine, using the computer software programs leading to explore protein formed by the alike spectra of a protein or DNA sequence data base [http://www.matrixscience.com/search-form-select.html]. The importance of similar protein spots with ion score was developed on the Mouse scoring algorithm (Pappin, 1993). Furthermore, ion score was measured by 10 × LOG [P], whereas P denotes as the complete prospectus which is detected similar match random event. However, a comparatively minor P mean values which will be the similar to be recognized protein and spectra (MS/MS) which is not in a random occurrence. A substantial specific match up surges the ion core and high score through greatly important matching protein spots [MASCOTHelp; http://www.matrixscience.com/

help/scoring_help.html]. In addition, an alone protein had a greater score than the lower score for the significance level at $p<0.05$ was adjudicated as a substantial similarity. In every MASCOT-search output outcome, the lower score for important limit was established on the total possibility and mass in order to database was being examined.

**Determination of leaf chlorophyll content and net photosynthesis rate:** The leaf chlorophyll values were taken after 45 days of transplanting by portable chlorophyll meter [MINOLTATM SPAD-502] (Peterson *et al.,* 1993). The SPAD-values were noted from the freshest fully extended leaf of rice plant. The comparative values expressed the unit of green color in plant leaf tissue that relates with the original values of chlorophyll contents in the plant tissues. The data was collected at many locations of similar leaf and standardization was compared with SPAD-value and leaf value per unit area by spectrophotometer. The single leaf net photosynthesis rate (Amax) was taken at 45day of planting from the YEL of every location utilizing LI-COR6200 Portable photosynthesis system, LI-COR-Inc Lincoln, Nebraska, USA. The data were collected in fully sunshine time with a continuous $CO_2$ of 380µmol $CO_2$ $mol^{-1}$ in the chamber.

**Statistical-analysis:** The data were statistically analyzed by the SAS-Software Program (Version-9.3), while treatments mean was isolated using Tukey's test-($p<0.05$).

## Results

**Soil chemical properties:** Soil was analyzed before experiment setup and it was acidic in nature (pH 4.5), non-saline (EC 1.12 dS $m^{-1}$) with CEC (5.24 $cmol_c kg^{-1}$). The organic carbon was 1.37%, total N 0.11%, Ca 0.60 $cmol_c kg^{-1}$, Mg 0.70 $cmol_c$ $kg^{-1}$, while exchangeable Al at the topsoil was slightly higher (1.13 $cmol_c kg^{-1}$). Generally soil was suitable for the aerobic rice cultivation.

**Effect of PGPR on protein expression**: Twelve 2-D gels were run to observe the plant enhancement by inoculation of PGPR. The spots of proteins were determined in the gels from the all replications. 2-D-PAGE analysis of leaf sheath protein showing varied expression of different protein spots that confirmed considerable alterations with great quantity linked with the control and soil amended treatments. The greatest dissimilar 12 differential spots were sequenced and then purposely categorized (Table 2).

**Differentially expressed proteins:** According to the functions of homologous proteins, 12 various proteins sequences were deposited [SWISSPROT]-and their accession numbers were collected from the GenBank organization. The data revealed that the spot1 (Genbank accession No-35172183) was obtained from the non-inoculated control. Such types of proteins were responsible for protein synthase into the plant (Table 2). Two proteins were expressed in Sb13 (*Bacillus* sp.) bacterial applied treatment, GenBank accession no. B8BL99 and I1P111 belonging to the protein and ribosomal protein L20P family, which can bind directly to 23S ribosomal RNA and are necessary for the general metabolism processes. However, Sb16 (*Stenotrophomonas maltophilla*) applied treatment had four proteins (GenBank accession no. B8BL99, gi1143427, Q9T0D3 & M0REW8). All proteins were different from each other and performed various functions. One of them was a light receptor which detentions and supplies excitation energy to the photosystems, belonging to the chlorophyll a and b binding LHC protein family, another two proteins were heat shock and heat stress transcription and the last one was disease resistance protein (Table 2).

The Sb42 (*Bacillus* sp.) applied treatment produced five proteins (GenBank accession no.K7KJ54, B0LT90, I1QKF7, 1HS13& M0VCA1). All of them varied from each other and performed different functions such as disease resistance *protein, phosphoric* diester hydrolase activity, lipid metabolic process, oxidation and reduction process, component of cytochrome b6-f complex, protein synthesizing functions. It binds directly to the 23S ribosomal RNA and other necessary metabolic processes in addition, the existence of the transgenes and decreased transcript excess, and plant stem area. It varied in cell wall thickness of xylem, fibers and the quantity of crystalline cellulose into the plant cell wall against the stresses and light receptor and supplies excitation energy into photosystems. It fixes at minimum 14 chlorophylls (8 Chl.a and 6 Chl.b) and carotenoids like lutein and neoxanthin (Table 2).

The PSB16 (*Bacillus* sp.) applied treatment also produced some important beneficial proteins. A total of five proteins were recognized with GenBank accession no. B8BL99, I0J180, I1QY78, 1HS13 and 35172183. However, some of the proteins showed homology with other PGPR strains, while some were differentially expressed (Table 2). These are mostly complicated in various important purposes in the light receptor, where they capture and deliver excitation energy to photosystems and belonged with chlorophyll a and b-binding (LHC) protein family. The second identified protein belongs to the ribosomal protein L20P family, binding directly to 23S ribosomal RNA and is necessary for 50S ribosomal subunit assembly processes. While, the remaining two proteins were ribosomal protein belonged toL20P family, which were binding directly to 23S ribosomal RNA and another necessary metabolic processes and existence of the transgenes. These transgenes decreased *BdCESA4* and *BdCESA7* transcript profusion and plant stem area, xylem cell wall thickness with fibers and the crystalline cellulose quantity (cell wall) in the plants against the stresses.

However, the application of bacterial consortium of PGPR showed the similar proteins found in the other treatments (Table 2). Based on the functions of homologous proteins, a total of six proteins were detected. Five of the proteins were similar to the proteins expressed by other PGPR, while one protein (*Populus trichocarpa)* with accession number (B9HQD5) varied and, that protein had the ability for the domain-containing transcription factor.

**Table 2. List of expressed differential proteins in rice leaf sheath tissues identified through 2-DE-LC-MS/MS.**

| Treatments | Spot | Accession number | Mows Score | Isoelectric point (PI) | MW (g/mol) | Identification of MS-blast | Protein organism | Functions of proteins |
|---|---|---|---|---|---|---|---|---|
| **Control** | 1 | 35172183 | 53 | 6.6 | 41844 | Tax_Id = 3847<br>Uncharacterized protein | *Glycine max* | beta-ketoacyl-acyl carrier protein synthase III |
| **Sb13** | 1 | B8BL99 | 110 | 6.15 | 53500.00 | Tax_Id= 39946<br>Putative uncharacterized protein | *Oryza sativa* sub sp. *indica* | The light receptor, it captures and delivers excitation energy to photosystems. Belongs to the light-harvesting chlorophyll a/b-binding (LHC) protein family |
| | 2 | I1P111 | 130 | 7.6247 | 55,564.62 | Tax_Id = 4538<br>Uncharacterized protein | *Oryzaglaberrima* | African rice protein gene. Ribosomal protein L20P family. Binds directly to 23S ribosomal RNA and other necessary metabolic processes |
| **Sb16** | 1 | B8BL99 | 110 | 6.15 | 53500.00 | Tax_Id = 39946<br>Putative uncharacterized protein | *Oryza sativa* sub sp. *indica* | The light receptor, it captures and delivers excitation energy to photosystems. Belongs to the light-harvesting chlorophyll a/b-binding (LHC) protein family. |
| | 2 | gi1143427 | | 5.1 | 75480 | Tax_Id = 3659<br>Malate dehydrogenase, cytoplasmic | *Cucumis sativus* | Heat shock protein 70 |
| | 3 | Q9T0D3 | 38 | 8.9 | 6234 | Tax_Id = 3702<br>HSFB2B B-2b | *Arabidopsis thaliana* | Heat stress transcription factor |
| | 4 | M0REW8 | 340 | 6.0406 | 30,439.17 | Tax_Id = 214687<br>Uncharacterized protein | *Musa acuminata* subsp. *malaccensis* | Disease resistance *protein. P*hosphoricdiester hydrolase activity. Lipid metabolic process, Oxidation-reduction process. Component of the cytochrome b6-f complex. |
| **Sb42** | 1 | K7KJ54 | 85 | 6.0406 | 30,439.17 | Tax_Id = 3847<br>Uncharacterized protein | *Oryza sativa indica* | Disease resistance *protein. P*hosphoricdiester hydrolase activity. Lipid metabolic process. Oxidation-reduction process. Component of the cytochrome b6-f complex. |
| | 2 | B0LT90 | 98 | | 60,000.00 | Tax_Id = 77588<br>Triosephosphateisomerase | *Oryza coarctata* | It is not involved in the protein synthesizing functions of that subunit, belongs to the ribosomal protein L20P family. (Wild rice) |
| | 3 | I1QKF7 | 87 | 7.6247 | 55,564.62 | Tax_Id = 4538<br>Uncharacterized protein | *Oryza glaberrima* | African rice protein gene. Ribosomal protein L20P family. Binds directly to 23S ribosomal RNA and other necessary metabolic processes |
| | 4 | 1HS13 | 150 | 6.5426 | 288,935.77 | Tax_Id = 15368<br>Uncharacterized protein | *Brachypodium distachyon* | Presence of the transgenes reduced *BdCESA4* and *BdCESA7* transcript abundance, as well as stem area, cell wall thickness of xylem and fibers, and the amount of crystalline cellulose in the cell wall in the plants against the stresses. |
| | 5 | M0VCA1 | 110 | - | 36354.00 | Tax_Id = 112509<br>Uncharacterized protein | *Hordeum vulgare* var. *distichum* | Light receptor and delivers excitation energy to photosystems. Binds at least 14 chlorophylls (8 Chl-a and 6 Chl-b) and carotenoids such as lutein and neoxanthin. |

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| **Sb13** | 1 | B8BL99 | 110 | 6.15 | 53500.00 | Tax_Id= 39946<br>Putative uncharacterized protein | *Oryza sativa* sub sp. *indica* | The light receptor, it captures and delivers excitation energy to photosystems. Belongs to the light-harvesting chlorophyll a/b-binding (LHC) protein family |
| | 2 | I1P111 | 130 | 7.6247 | 55,564.62 | Tax_Id = 4538<br>Uncharacterized protein | *Oryzaglaberrima* | African rice protein gene. Ribosomal protein L20P family. Binds directly to 23S ribosomal RNA and other necessary metabolic processes |
| **Sb16** | 1 | B8BL99 | 110 | 6.15 | 53500.00 | Tax_Id = 39946<br>Putative uncharacterized protein | *Oryza sativa* sub sp. *indica* | The light receptor, it captures and delivers excitation energy to photosystems. Belongs to the light-harvesting chlorophyll a/b-binding (LHC) protein family. |
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| **Sb42** | 1 | K7KJ54 | 85 | 6.0406 | 30,439.17 | Tax_Id = 3847<br>Uncharacterized protein | *Oryza sativa indica* | Disease resistance *protein. P*hosphoricdiester hydrolase activity. Lipid metabolic process. Oxidation-reduction process. Component of the cytochrome b6-f complex. |
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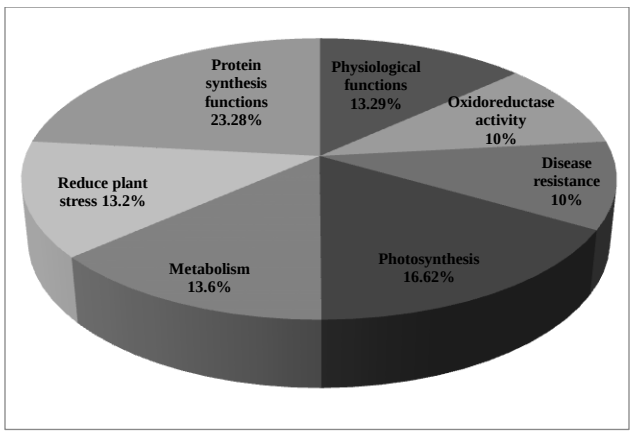


Fig. 1. Assignment of the identified proteins to functional categories using the classification method described by Bevan *et al.*, (1998). A total of 153 spots representing 12 different proteins were classified.

In our study 12 proteins were known having different potential and beneficial characteristics (Fig. 1). Among these 23.28% of the proteins were responsible for protein synthesis, 13.29% were controlling physiological functions, 16.62% proteins were performing for photosynthetic activities, 13.6% proteins were involved in metabolic process, while 13.2% were participated against the plant stress and physiological functions whereas,10% disease resistant and 10% oxidoreductase activity, respectively.

**Effects PGPR application on the chlorophyll values in rice:** Leaf chlorophyll content and net photosynthesis rate were measured which varied with treatments (Table 3). Generally application of PGPR alone or in consortium increased the chlorophyll contents (SPAD values) and net photosynthesis rate in the leaves of rice. Significantly ($p$<0.05) greater contents of chlorophyll (45.60) and photosynthesis rate (8.34 μmol $CO_2$ $m^{-2}$ $s^{-1}$) were observed with bacterial consortium treatment. High chlorophyll contents and photosynthetic activity in the inoculated leaves related to higher contents of the nitrogen that may lead to the improved plant growth and yield.

**Effects of PGPR application on growth of rice:** The application of PGPR positively affected plant growth (Table 4). The growth of rice plants was significantly enhanced with all PGPR treatment compared to the control. The greater plant dry biomass (10.16g), plant-height (92 cm), root-length (27 cm) and tillers numbers (6) were observed in the bacterial consortium applied treatment.

**Effects of PGPR application on the plant nutrient uptake and protein:** The PGPR application increased the plant nutrient and protein values in the rice grain. The higher nutrients concentration in grains was observed in PGPR applied treatment (Table 5). However, the highest N (0.61%), P (0.14 %), K (1.49 %) and protein contents (6.17 %) were found in the mixed bacterial consortium.

**Table 3. Effect of PGPR inoculation on leaf chlorophyll content and photosynthesis of aerobic rice at 45 days of planting.**

| Treatments | Chlorophyll content (SPAD values) | Photosynthesis (μmol $CO_2$ $m^{-2}$ $s^{-1}$) |
|---|---|---|
| Control | 34.10d | 6.02d |
| Sb13 (*Bacillus* sp.) | 38.57c | 6.98c |
| Sb16 (*Stenotrophomonas maltophilla*) | 42.23b | 7.74b |
| Sb42 (*Bacillus* sp.) | 39.00c | 7.52b |
| PSB16 (*Bacillus* sp.) | 41.32b | 7.68b |
| Bacterial consortium | 45.60a | 8.34a |

Means within the same column followed by the same letters are not significantly different at $p$<0.05

**Table 4. Effects of PGPR inoculation on the vegetative growth of aerobic rice at 45 days of planting.**

| Treatments | Plant biomass plant$^{-1}$ (g) | Plant height (cm) | Root length (cm) | Tillers plant$^{-1}$ |
|---|---|---|---|---|
| Control | 7.3d | 81c | 22c | 4c |
| Sb13 (*Bacillus* sp.) | 9.2b | 90a | 27a | 6a |
| Sb16 (*Stenotrophomonas maltophilla*) | 8.6c | 85b | 24b | 5b |
| Sb42 (*Bacillus* sp.) | 8.5c | 90a | 24a | 5b |
| PSB16 (*Bacillus* sp.) | 9.1b | 87b | 26b | 6a |
| Bacterial consortium | 10.6a | 92a | 31a | 6a |

Means within the same column followed by the same letters are not significantly different at $p$<0.05 (n = 4)

**Table 5. Effect of PGPR inoculation on the nutrient and protein content in aerobic rice grain at harvest.**

| Treatments | N | P | K | Total protein content |
|---|---|---|---|---|
| | ---------- % ---------- | | | |
| Control | 0.42d | 0.09c | 1.36c | 5.59d |
| Sb13 (*Bacillus* sp.) | 0.51c | 0.10b | 1.43b | 5.80c |
| Sb16 (*Stenotrophomonas maltophilla*) | 0.57b | 0.12b | 1.46b | 5.97b |
| Sb42 (*Bacillus* sp.) | 0.54c | 0.11b | 1.44b | 5.77c |
| PSB16 (*Bacillus* sp.) | 0.53c | 0.13a | 1.45b | 6.01b |
| Bacterial consortium | 0.61a | 0.14a | 1.49a | 6.17a |

Means within the same column followed by the same letters are not significantly different at $p$<0.05 (n = 4)

## Discussion

Application of PGPR improved plant growth of rice cultivar MR219-9. The proteins involved in growth promotion and plant-microbe interactions were investigated by analyzing 2-D protein profiles. There were about153 protein spots developed by inoculated rice plants. Amongst them 12 different proteins spots were determined with either up or down regulated and were involved in the growth promotion of rice plants. Plants treated with the bacterial consortium exhibited greater number of protein spots in leaf sheath of rice having a variable spot sizes. Many identified proteins presented divergence among their experimental and predictable molecular weight, and these results are similar to those of earlier work done by Bedon *et al.,* (2011). The main reason could be mainly by protein degradation with lower experimental molecular weight and post translational changes (Plomion *et al.,* 2006). Moreover, proteins are the carriers and perform significant functions in rice leaf cells. These proteins were expressively affected by the variations in cell function or intercellular environmental effects at various growth stages (Shao *et al.,* 2008).

The result of 2-D protein profiling discovered the important proteins involved in the various important physiological functions into the system of the plant that lead to plant growth promotion. These protein spots were identified and found to have significant roles in various functions in the plants like ribosomal activity performed directly to 23S ribosomal RNA, metabolic processes with participating significant role in chlorophyll and photosynthesis, and some of the proteins performed a vital role for disease and biotic resistances against the plant stress. These proteins were produced by the different PGPR for the plant defense system and growth enhancement. In this study, the 2-D analysis exposed various proteins similar to that found in vegetative tissues of *P. chinensis.* Similarly, some important spores were matched (MASCOT score69) including 6 various efficient proteins from rice such as Arabidopsis pearl millet, castor oil plant, tomato and salt cedar (Xiong *et al.,* 2013).

The various types of proteins including some of wild species were proposed as a prospective pool of genetic dissimilarity for the crop growth. Protein encoding genes have ability to defend the disease infestation and simultaneously enhance the resistance against disease. While, some of the protein species (wild species) played positive role for crop growth increment and other yield related components (Mahmoud *et al.,* 2007).

The identified protein spots participated important functions for the plant stress and controlling the cell death through preservation of responsive oxygen homeostasis species. The aerobic rice plants comparatively remained under water stress, therefore these proteins might be the reason to tolerate plant drought stress. Similarly the previous results were reported by Yeh *et al.,* (2011) that protein (BohLOL1) was involved in bamboo development in relation to biotic stress. However, existence of proteins comprising glutelins creates *B. distachyon* quicker in rice than the wheat endosperm (Larré *et al.,* 2010).

The application of various potential PGPR in the rice plant improved the chlorophyll contents and photosynthesis activity. Predominantly the bacterial consortium significantly ($p<0.05$) increased the chlorophyll contents and photosynthesis similar effects to that observed by Panhwar *et al.,* (2011). Furthermore PGPR positively affected growth of rice plants and increased the nutrient uptake through the beneficial mechanisms such as plant growth hormones, organic acids, nitrogen fixing and P solubilizing abilities (Panhwar *et al.,* 2012). These beneficial effects were supported through identification of protein spots which bear similar characteristics. In addition, the infertility of soils could be improved by the microbes used as bio-organic fertilizer application in rice (Alia Farhana *et al*., 2016).

The PGPR used showed their beneficial potential on the rice plants and produced various types of proteins. The proteins spots perceived in current research were recognized according to their functions and mostly responsible for metabolic, disease resistance, various plant stresses and remaining other physiological functions. Proteins play some important functions inside plant system which is responsible for the plant defensive mechanism system as well as protection in contrast to plant-stress and other metabolic processes-(Kim *et al.,* 2004). Additionally, these proteins can also be involved in several unknown functions which have to be investigated and the proteomics approach clarifies new research locations for monocot cell walls (Douché *et al.,* 2013; Handakumbura *et al.,* 2013).

## Conclusion

It is therefore, concluded that the study delivers new imminent mechanisms about the PGPR application that improved the aerobic rice growth through their beneficial traits. The proteomic study showed 12 protein spots extracted from the rice leaf sheath, which many of them were responsible in controlling abiotic stress, disease resistance, and oxidation reduction process, protein synthesizing functions and photosynthesis related physiological process. Application of PGPR changes cellular protein that affects the molecular structure of rice plants leading to improve aerobic rice growth.

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