Studying the effect of Plant Growth Promoting Rhizobacteria supplementation on growth and seed yield of *Brassica campestris* L. (mustard plant)

Thesis submitted to the University of North Bengal
For the Award of Doctor of Philosophy in Biotechnology

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DECLARATION

I hereby declare that the thesis entitled "**Studying the effect of Plant Growth Promoting Rhizobacteria supplementation on growth and seed yield of *Brassica campestris* L. (mustard plant)**" has been prepared by me under the guidance of Dr. Shilpi Ghosh, Associate Professor, Department of Biotechnology, University of North Bengal.

No part of the thesis has formed the basis for the award of any degree or fellowship previously.

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I certify that Ms. DeepikaMazumdar has prepared the thesis entitled "**Studying the effect of Plant Growth Promoting Rhizobacteria supplementation on growth and seed yield of *Brassica campestris* L. (mustard plant)**" for the award of Ph.D. degree of the University of North Bengal under my guidance. She has carried out whole work in the department of Biotechnology, University of North Bengal. No part of the thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

The 20th century green revolution transformed agriculture with a massive gain in global production of food. A major effort of green revolutions comprised the use of new chemical fertilizers, including nitrogen (N) fertilizers. However, increase in agricultural production at the expense of application of higher quantities of N-fertilizers has been realized to cause enormous environmental hazards and thus imposing doubts on sustainability of food production. Hence, newer and sustainable agricultural approaches of enhanced food production with minimum use of chemical fertilizers are highly desired. In true sense, phytomicrobiome including PGPR, can play vital role in 21st century green revolution due to their key roles in nutrient acquisition and assimilation, improved soil texture and modulating extracellular molecules, like hormones and other signalling molecules, and improved stress tolerance, all leading to enhancement in plant growth. However, bacterial formulations with PGPR have not always the desired effectiveness. Keeping these in views, present investigation aimed to develop nutrient formulation (NF) containing PGPR with reduced N input for optimum plant growth promotion. For achieving the objective of the study, initially 82 bacterial strains isolated from rhizospheric soil. The isolates initially screened on the basis of their ability to fix atmospheric N. In total 27 isolates were found to fix nitrogen, which were further checked qualitatively and quantitatively for the other plant growth promoting trait. Among the N fixing isolates, 12 produced Indole acetic acid (IAA) (44%), 22 solubilised dicalcium phosphate (DCP) (81 %), 6 solubilised tricalcium phosphate (TCP) (22%), 16 produced phytase (59%), 14 and 16 isolates showed zone of solubilisation in media with zinc phosphate (52 %) and zinc carbonate (59 %), respectively, 11 isolates showed positive for ACC deaminase (40%). All the 27 isolates when checked for quantitative ammonia production in Asbhy's N free broth medium, the isolates RS3, RS23, RS26 and RS51 produced more than 6 µg mL^{-1} of ammonia with significantly higher production by RS3 (9.52 µg mL^{-1}) and RS26 (10.13 µg mL^{-1}). The liberation of Pi by RS3, RS23, RS26 and RS49 were recorded to be 49, 40, 26 and 30 µg ml^{-1}, respectively. Significantly higher phytase activity of RS3, RS23, RS26 and RS49 were recorded in the pH range 4.5 to 5.5. The isolates RS3, RS9 and RS26 showed Zinc solubilisation index (ZSI) on Zinc carbonate of 2.34, 2.06 and 2.07, respectively. Bacterial strains RS3 and RS26 produced significantly greater quantity of IAA and their respective production levels were 5.06 and 7.13 µg mL^{-1} in absence of tryptophan and 10.13 and 14.51 µg mL^{-1} in presence of tryptophan. RS2, RS3, RS6, RS10, RS16, RS26, RS31, RS53, RS59, RS60 and RS65 were ACC deaminase positive. Principal Component Analysis (PCA) of the PGPR traits of the isolates was used to select the potent PGPR strains for pot experiment. PCA extracted two components, PC1 and PC2 which explain 32.7 and 30.1 % variability, respectively. The microbial strains RS3 and RS26 contributed to PC1 with a correlation coefficient of 0.607 and 0.602, respectively. PCA thus suggested RS3 and RS26 as potent PGPR. The phylogenetic analysis based on 16S rRNA gene sequence identified isolate RS3 as *Cedacea davisae* RS3 (GenBank accession number KX101223) and the strain RS 26 as *Klebsiella pneumoniae* RS26 (Gene bank Accession number MH 819506.1). The

two isolates showed compatibility with each other and were negative in hemolysin production test in sheep blood agar medium.

Further, nutrient formulations containing PGPR (*Cedacea davisae* RS3 and *Klebsiella pneumoniae* RS26) and varying N levels were formulated by following general and central composite rotatable design (CCRD) based response surface methodology (RSM) approaches and their effects on plant growth and yield were compared. In general approach, mustard plants were grown in four different NF treatment regimens, namely, N-appropriate without microbes (N^+PGPR^-), N-appropriate with microbes (N^+PGPR^+), N-deficit without microbes (N^-PGPR^-), N-deficit with microbes (N^-PGPR^+) and their growth characteristics were compared. Plant under N^+PGPR^- NF showed the highest seed yield (5.76 g plant-1), protein content of root (378.67 mg g^{-1} FW) and shoot (675.89 mg g^{-1} FW), the carbohydrate content of root 6.76 (mg g^{-1} FW) and shoot (12.17 mg g^{-1} FW). The other parameter like number of siliqua per plant (39.40), number of seeds per siliqua (30.12), 100 seed weight (0.48 g) and seed yield (5.76 g $plant^{-1}$) at 75 DAT were also maximum in N^-PGPR^+ treatment group. In RSM based approach, the effect of three variables, N concentration (A), inoculum volume of strain1 i.e. *C. davisae* RS3 (B) and inoculum volume of strain 2 i.e. *K. pneumoniae* RS26 (C) of the NF, on plant growth (carbohydrate and protein content of shoot) and seed yield was investigated, using Central composite rotatable design (CCRD). The experimental runs suggested that the application of NF containing N at 0.5 mM and strain 1 and strain 2 at 50% v/v each to the cultivation of mustard plant, yielded actual response of shoot carbohydrate content (16 mg g^{-1}FW), shoot protein content (824 mg g^{-1}FW) and seed yield (8.10 g $plant^{-1}$), which are quite close to the model predicted response comprising shoot carbohydrate content of shoot (15.04 mg g^{-1} FW), shoot protein content (819.25 mg g^{-1} FW), seed yield (8.20 g $plant^{-1}$).

A comparison of the effect of general and RSM based approaches of plant NF treatments to the growth and yield of mustard plant indicated that carbohydrate and protein contents and seed yield, GS activity (211 µmole g^{-1} FW) and Chlorophyll a (2.7 mg g^{-1} FW) of RSM based treatment plants were significantly greater than that of the plant treated with general approach with respect to the carbohydrate content, protein contents, seed yield, GS activity (121 µmole g^{-1} FW) and Chlorophyll a content (2.3 mg g^{-1} FW). The results herein suggest that RSM based optimization of NF could be beneficial for enhanced plant growth and yield leading to agricultural sustainability.

To gain an insight into the role of NFs in plant growth, mustard plants treated with NFs showing highest yield (designated as 'treated') was subjected to transcriptomic based differential gene expression (DGE) analysis using plants treated with NF containing optimum level of inorganic N as control (designated as 'control'). The DGE analysis showed that 25,088 protein coding genes were expressed in both control and treated group of plant, where as 357 genes were exclusively expressed in treated group only, 351 genes were exclusively expressed in control group of plants only. The expression level of total 556 genes were found to be up regulated and 690 genes were found to be down regulated as compared to the control group. Gene ontology annotation associated with upregulated, downregulated, expressed both and exclusively expressed genes for the sample combination control vs treated were obtained. In case of treated plants, among the cellular components and biological processes, the expression of genes related to organelle,

extracellular region part, localization, cell proliferation, detoxification, carbon utilization was increased, whereas genes encoding proteins for nucleiod and rhythmic processes were found to be downregulated. Metabolic pathways related to plant hormone signalling and antenna protein mediated photosynthesis potrayed the expression of the genes related to phytohormone signaling and light harvesting genes were also upregulated. Five genes associated with the phytohormone signal transduction pathway were found to be elevated. They encoded Auxin-responsive GH3 family protein (GH3), SAUR-like auxin-responsive protein family, ABA-responsive element binding factor (ABF), Sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK2), and basic-leucine zipper (bZIP) transcription factor family protein and three genes of antenna protein mediated photosynthesis namely Lhca1 (Chlorophyll a/b binding protein 6), Lhcb1 (PSII Light harvesting complex protein 1), Lhcb2 (PSII Light harvesting complex protein 2) were upregulated.

Application of RSM optimized NF to the plant resulted in upregulation of several genes encoding proteins or enzymes to be directly associated with plant productivity like nitrate reductase, ammonia transporter, amino acid transporter family protein, inorganic phosphate transmembrane transporter, flowering promoting factor 1, seed storage/lipid Transfer Protein (LTP) family protein, mitochondrial phosphate transporter etc. Furthermore, several gene products which indirectly affect plant performances by giving resistance against biotic and abiotic stress were also upregulated, glutathione peroxidase, Lys/His transporter 7, UDP-glucosyl transferase family protein, glutathione S-transferase F3, mildew resistance locus O12, INH3, monooxygenase, disease resistance response and pleiotropic drug resistance 7, phenylalanine ammonia-lyase, WRKY35 transcription factor, NADP-dependent oxidoreductase, chorismate mutase 1, CTP synthase, growth regulating factor 2, 1, transmembrane transporter and $NADP^+$ isocitrate dehydrogenase. Those genes whose downregulation have significant role in plant growth and yield found in this study were cell wall / vacuolar inhibitor of fructosidase 1, WRKY18 trancription factor, pyruvate decarboxylase, senescence-associated protein-related and glutamine dumper 1.

Thus, *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26 proved to be potent PGPR and the RSM approach can be a promising tool for designing the nutrient formulations containing reduced N input with PGPR supplements for enhanced plant growth and yield. Such type of work, not only increase crop productivity without affecting the environment but is also cost effective. Hence, this effort can put a small building block in the development of sustainable agriculture.

PREFACE

Since the dawn of civilization on planet earth, agriculture has been considered the most important resource for maintenance of livelihood of the mankind/ financial source of the mankind. Therefore, the dynamic soil nature of crop field is the main focus/for sustainable agriculture. In India more than 60% of its land is used for agriculture to cultivate several types of vegetables, cereals, pulses, oilseeds, fruits etc. Organic carbon content, moisture contents, minerals like, phosphorus, nitrogen, potassium, and other different biotic and abiotic factors are important which regulates the soil quality for crop production. However, the soil pH and exchangeable bases are reduced and soil nutrients become unavailable to crops due to the indiscriminate use of chemical fertilizers, especially nitrogen and phosphorus fertilizers. As the agricultural land quantity is limited and world population is increasing at an alarming rate, which in turn is generating tremendous pressure for the overproduction of edible crops. Hence, to fulfil the increasing food demand through maintaining the soil fertility is highly warranted, which urgently require improved scientific farming techniques. Genetically engineered crops, agricultural intensification, sustainable management practices, use of genetically modified microbes in the crop field and use of biofertilizers are some of the current techniques that are being used for sustainable agriculture. The desired goal can also be fulfilled by the use of soil microorganisms, such as fungi, algae and bacteria which can promote plant growth. Association of microbes to the plants can be best described by plant growth promoting rhizobacteria (PGPR), which by various synergistic mechanism help not only to induce plant growth but also give protection to the host plant from pathogens. For conversion of barren poor-quality land into fertile cultivable land, PGPR plays crucial role. The research work presented herein describe the effect of PGPR supplementation to the NF with varying input of N on plant performance. For achieving the objective, the mustard plants were treated with the NF having differential N inputs and two PGPR isolates *C. davisae* RS3 and *K. pneumoniae* RS26, employing both the general and statistical approaches and their effect on plant growth and yield were compared by monitoring morphological and biochemical parameters, and seed yield. The plants under NF treatment regime showing highest growth and yield was subjected to differential transcriptomics analysis using plants treated with optimum level of inorganic N as control.

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"Gratitude turns what we have into enough, and more. It turns denial into acceptance, chaos into order, confusion into clarity...it makes sense of our past, brings peace for today, and creates a vision for tomorrow''- Melody Beattie. With this beautiful quotation, I would like to go back to my past and will try to mention about some people with whom I came across and who really changed my way of thinking and doing.

To those people in my life who are the source of inspiration and motivation to me throughout this journey, I want to express my deepest thanks. At the very first, I would like to bestow my gratitude to my supervisor, **Dr. Shilpi Ghosh** for giving me the opportunity to work in her laboratory. Any word of gratitude will be very small as because when I first joined the laboratory, I was a mere amateur who has no practical knowledge of biotechnological techniques. Still she believed on me and allowed me to work in her UGC project. She trained me with the techniques of Molecular Biology and Biochemistry and excel the good in me. Her hardworking, sincerity, ambitious and administrative nature has always amazed me and inspired me to be like her. Her appreciation and constructive suggestions during the planning and development of this research work have installed in me that there is no limit to perfection and "The more we learn and practise, the more we become perfect". Whatever I learnt here all because of her and Iam very much lucky to have her as my supervisor.

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Siblings share one of the best relationships of all. Whether it is between a brother to sister or between sister to sister, we cannot deny the importance of such a relationship where love dominates all the time. And so I want to show my gratitude to my brother **Mithu Mazumdar** and sister **Mamta Mazumdar** loving me eternally. I would also like to thank my entire family with my mother-in Law **Smt Chhaya Roy Saha** for her constant motivation and brother-in-law **Surajit Bhattacharjee**, sister-in-law **Mahuya Mazumdar** and lovely niece **Navya Mazumdar** for being the reason of my strength to overcome the odd situations during this journey.

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I want to bestow my gratitude to my grandparents (**Late Jitendra Mazumdar**, **Late Parul Mazumdar**, **Late Gopal Sarkar**, **Late Mukul Sarkar** and uncle **Late Manik Sarkar**) for always giving me love, affection and appreciation. I wish they can see me and bless me from wherever they are. Last but not least, I am grateful to **Almighty God** for giving me good health and happiness that were necessary to complete this journey. He gives me the power of patience and tolerance immensely. I bestow my whole hearted thanks to Him for surrounding me with beautiful and inspirational human beings. And I also pray to Him to give good health and fortune to everyone who helped me in any way.

Thank you everyone

Deepika Mazumdar 8/9/2020

Deepika Mazumdar

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CHAPTER ONE

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

1.1. Introduction

In the present era, one of the major global issues is world food hunger. There is a continuous demand of food supply for the world population which is increasing at an alarming rate. The Declaration of the World Summit on Food Security (FAO, 2009) calls for an average annual increase in food production of 44 million metric tons to feed approximately 9 billion people by 2050 (Godfray et al., 2010). It is well known that sustained crop productivity relies on constant supply of nutrients which is mainly applied in the form of inorganic fertilizers containing phosphate, nitrate, ammonium and potassium. In the last five decades, the rate of nitrogen, phosphorus, and potassium (NPK) fertilizer application has increased tremendously. The International Fertilizer Industry Association reported that the three countries with the highest fertilizer use in 2006 were China, India, and USA, consuming 50.15, 21.65, and 20.83 million tons of NPK fertilizer, respectively, compared with consumption in 1961 of 1.01, 0.42, and 7.88 million tons, respectively. Although the application of chemical fertilizers has enhanced soil fertility and crop productivity, it often negatively affect the complex system of biogeochemical cycles, including nitrate leaching into ground water, run-off of nitrogen and phosphorous, and eutrophication of aquatic system (Smolders et al., 2010). Therefore, integrated nutrient management systems are needed to maintain agricultural productivity and protect the environment. Due to the emerging demand for reduced dependence on synthetic chemical products and for the growing necessity of sustainable agriculture within a holistic vision of development and environmental protection, the plant growth promoting rhizobacteria (PGPR) have gained worldwide importance and acceptance for their agricultural benefits.

PGPR are the soil bacteria inhabiting around/on the root surface and are directly or indirectly involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of rhizosphere. The term PGPR includes three types of soil bacteria, depending on their lifestyle: free-living bacteria inhabiting the zone around the root (rhizosphere), bacteria that colonize the root surface (rhizoplane), and endophytic bacteria that live within roots. However, this division is not exclusive, since any individual bacterial strain may adopt any of the three life strategy depending on the soil environment conditions and the host-root partner involved (Alavi et al., 2013; Mitter et al., 2013). PGPR enhance plant growth and development by a number

of mechanisms. They have either direct mechanisms to facilitate uptake of nutrient, availability of nutrient through nitrogen fixation, solubilisation of complex organic nutrients, and production of phytohormones or indirect mechanisms to produce antibiotics, siderophores, hydrogen cyanide (HCN), hydrolytic enzymes etc. (Ahemad and Kibret, 2014). Although the plant growth promotion capacity of PGPR can be more easily determined under controlled conditions using sterile substrates, the inoculated PGPR in soil may compete with other microflora with loss of their positive effects (Sturz and Christie, 1995).

Crop productivity has fundamental dependence on inorganic nitrogen (N) fertilization. During the past five decades, the application of N fertilizers has resulted in greatly increased global food production and decreased world hunger. According to the report of the United Nations Food and Agricultural Organization, the World population under synthetic N-fertilizer is continuously increasing and in 2015, out of the total world population of 7.38 billion, 3.54 billion population were supported by synthetic fertilizers (Fig 1.1).

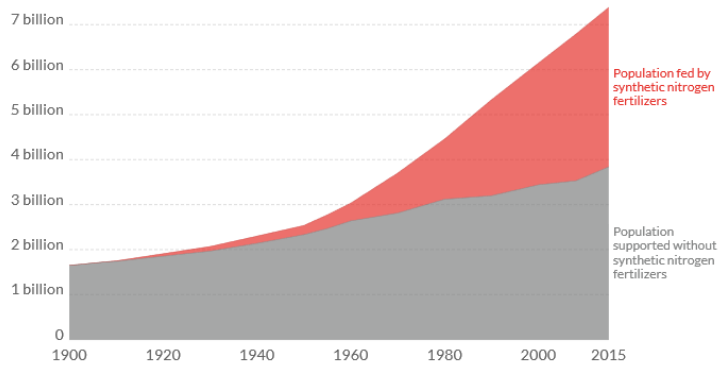

Image courtesy: Max Roser and Hannah Ritchie (2020)

Fig 1.1. World population supported with and without synthetic nitrogen fertilizers

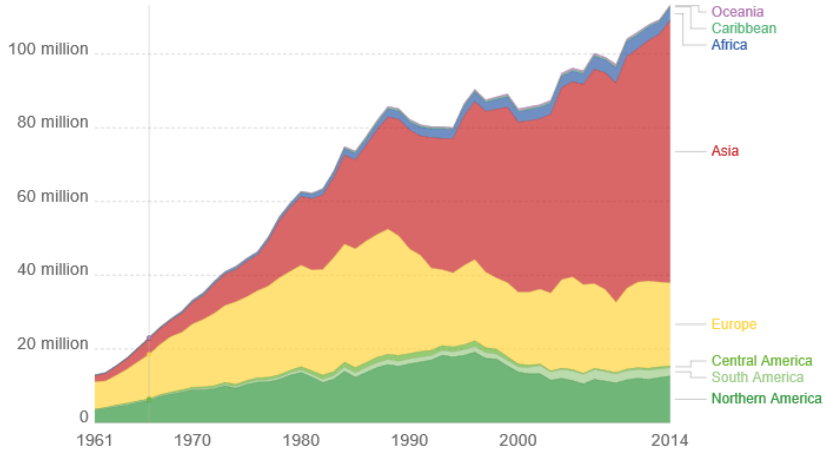

Image courtesy: UN food and agricultural organization

Fig 1.2. Global Nitrogen fertilizer production (tonnes of nitrogen produced per year)

The global production of N-fertilizers increased from 80 million tonnes per year in 2000 to more than 100 million tonnes per year in 2014 (Fig 1.2). Approximately 100 million metric tons (MMt) of nitrogenous fertilizers are added to soil worldwide annually, which is predicted to increase to 240 MMt by the year 2050 (Tester and Langridge 2010; Sharma et al., 2017). Consequently, world population fed by plants grown on synthetic nitrogenous fertilizers has increased considerably. Currently, the application of N fertilizers in India has reached to more than 100 kg per hectare of cropland (Fig 1.3) (http://www.fertilizer.org/ifa).

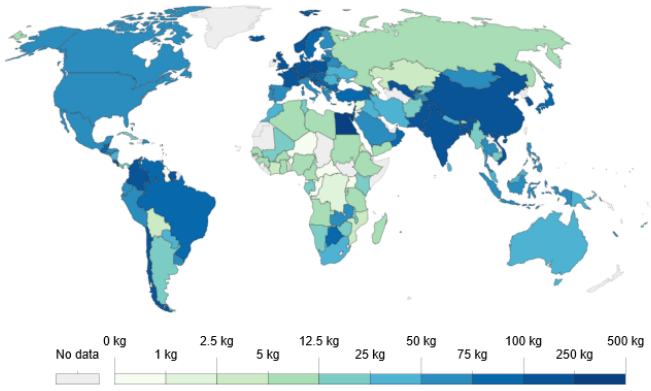

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Fig 1.3. Nitrogen fertilizer use per hectare of cropland, 2017 (Kg of total nutrient per hectare)

The increased application of inorganic nitrogenous fertilizers in agriculture has economic consequence because it is one of the major costs associated with the production of high-yielding crops. Moreover, the excess use of N-fertilizers causes significant environmental damage due to their inefficient uptake by the plants and as a result only 30 to 65% of the applied N being utilized. Excess N compounds released from agricultural systems as N_2, trace gases or leached nitrate threaten the quality of air, water, and soil. Increased soil leaching into drainage water and the release of atmospheric nitrous oxide and reactive N gases (NO*x*) into the troposphere can lead to acidification of soils, eutrophication of waterways and death of aquatic life (Ottman and Pope, 2000; Diaz and Rosenberg 2008; Xu et al. 2012). Moreover, nitrate (NO_3^-**)** that accumulates in harvestable vegetative organs has been considered as a source of potential danger to human health.

Present study was undertaken with the aim to reduce the inorganic N input in soil with the aid of PGPR with enhancing or maintained plant yield. The research work involved isolation of PGPR from rhizospheric soil followed by selection of two potent PGPR for application to mustard (*Brassica campestris* L.) plants. For plant applications the nutrient formulations (NF) containing various levels of N and PGPR, were optimised by general method and by the statistical tool, namely, Response Surface Methodology (RSM). The plants treated with the NFs were evaluated for morphological and biochemical and yield parameters. Finally, the RSM optimised NF treated plant with highest yield was subjected to transcriptomic analysis.

1.2. Objectives

The scope of the investigation was confined to the following objectives:

1. To isolate bacteria from rhizospheric soil.
2. To determine plant growth promotion (PGP) traits of rhizobacterial isolates and their morphological and biochemical characterization.
3. To screen the potent plant growth promoting rhizobacteria (PGPR) on the basis of qualitative and quantitative tests of various plant growth promoting (PGP) attributes.
4. Phylogenetic characterization of the potent PGPR.
5. To formulate general and RSM based nutrient formulations containing potent PGPR and various levels of nitrogen and application to mustard plant.
6. To evaluate the performances of the plants under various treatment regimes by morphological, physiological and biochemical parameters.
7. Comparative transcriptomic analysis of mustard plant roots.

1.3. Review of Literature

This chapter is an effort to elucidate the concept of PGPR in the current scenario and their underlying mechanisms of plant growth promotion with recent updates. The latest paradigms of a wide range of applications of these beneficial rhizobacteria in different agro-ecosystems have been presented explicitly to garner broad perspectives regarding their functioning and applicability.

1.3.1. Rhizosphere

Architecture of the root plays a crucial role in plant uptake of water, nutrients and minerals and to provide anchorage in the soil. The root is a dynamic structure with growth and branching depending on the continuous incorporation of internal and environmental factors (Asari et al., 2016). The narrow zone of soil directly surrounding the root system is known as rhizosphere (Walker et al., 2003). It is a nutrient rich habitat that harbours a huge variety of microorganisms having `neutral, beneficial or deleterious effects on the plant. The term 'rhizobacteria' implies a group of rhizospheric bacteria competent in colonizing the root environment (Kloepper et al., 1991). Plant roots synthesize, accumulate, and secrete a diverse array of compounds (Table 1.1) as root exudate, which act as chemical attractants for a vast number of heterogeneous, diverse and actively metabolizing soil microbial communities (Walker et al., 2003). The composition of root exudate is dependent upon the physiological status and species of plants and microorganisms (Kang et al., 2010). The root exudate carry out wide range of chemical and physical modifications to the soil; and acts as chemo-attractant or chemo-repellent and hence, regulate the diversity of actively metabolizing soil microbial communities (Walker et al., 2003) as well as the structure of microbial community in the immediate vicinity of root surface (Dakora and Phillips, 2002). Moreover, these exudates support the beneficial symbiotic interactions of plants and microbes (Nardi et al., 2000). On the other hand, microorganisms present in the rhizosphere determine the plant rooting patterns and nutrient availability, thereby modifying the quality and quantity of root exudates. They also metabolize a fraction of plant-derived small organic molecules in the vicinity as carbon and nitrogen sources, and some microbe-oriented molecules are subsequently absorbed up by plants for growth and development (Kang et al., 2010). In a previous report, Marschner (1995) reported that carbon fluxes of plants are critical determinants of rhizosphere function and approximately 5–21% of photosynthetically fixed carbon is transported to the rhizosphere through root exudation process. Hence, the rhizosphere can be defined as any volume of soil which is influenced by plant roots and/or in association with roots hairs, and plant produced compounds (Dessaux et al., 2009). Furthermore, three large and separate but interacting components are recognized in the rhizosphere namely, the rhizosphere, the rhizoplane, and the root itself. Of these, the rhizosphere is that zone of soil in association of roots that

affect microbial activity by the release of substrates. The rhizoplane, on the other hand, is the root surface including the strongly adhering soil particles while the root itself is a component of the system, because many micro-organisms like endophytes also colonize the root tissues (Barea et al., 2005).Microbial colonization of the rhizoplane and/or root tissues by the microbes is known as root colonization, whereas colonization of the adjacent volume of soil under the influence of the root is known as rhizosphere colonization (Barea et al.,2005; Kloepper et al., 1991; Kloepper, 1994). In a broad aspect, these can be separated into extracellular (ePGPR) existing in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex, and intracellular (iPGPR), which exist inside root cells, generally in specialized nodular structures (Figueiredo et al., 2011). Some examples of ePGPR include A*grobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcous*, *Pseudomonas* and*Serratia* etc. (Bhattacharyya and Jha, 2012). Similarly, some examples of the iPGPR are *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* belonging to the family Rhizobiaceae.

Table 1.1.
Chemical compounds present in root exudates from various plant species

Types of chemical compounds	Names of the compounds
Sugars	Glucose, Galactose, Fructose, Xylose, Maltose, Rhamnose, Arabinose, Raffinose, Deoxyribose, Ribose
Amino acids	α-Alanine, β-alanine, Glycine, Asparagine, Aspartate, Cystein, Cystine, Glutamate, Isoleucine, Leucine, Lysine, Methionine, Serine, Threonine, Proline, Valine, Tryptophan, Ornithine, Histidine, Arginine, Homoserine, Phenylalanine, γ-Aminobutyric acid, α-Aminoadipic acid
Organic acids	Citric acid, Oxalic cid, Malic acid, Fumaric acid, Succinic acid, Acetic acid, Butyric acid, Valeric acid, Glycolic acid, Piscidic acid, Malonic acid, Formic acid, Aconitic acid, Lactic acid, Glutaric acid, Tetronic acid, Aldonic acid, Erythronic acid, Pyruvic acid,
Vitamins	Biotin, Thiamine, Pantothenate, Riboflavin, Niacin
Enzymes	Acid/alkaline phosphatase, Invertase, Amylase, Protease
Purines /nucleosides	Adenine, Guanine, Cytidine, Uridine
Inorganic ions and gaseous molecules	HCO_3^- , OH^-, H^+ CO_2.H_2, CO_2, H_2O
Secondary metabolites	Phenolics, Flavonoids, Terpenoids, Jasmonicacid, Salicylic acid, Brassinosteroids, Auxins, Cytokinins, Gibberellins, Ethylene and Abscissic acid

1.3.2. Plant Growth Promoting Rhizobacteria (PGPR)

PGPR are the soil bacteria that are found predominantly around or on the root surface and are directly or indirectly involved in promoting plant growth and development via multiple plant growth promoting mechanisms. In 1909, Bottomley firstly reported the use of soil bacteria to promote plant growth apart from in a *Rhizobium*–legume symbiosis. The study showed that a consortium of *Pseudomonas radicicola* and *Azotobacter* sp. increased the the yield of barley (*Hordeum vulgare* L.), growth of oat (*Avena sativa*), and the bulb weight of summer hyacinth (*Galtonia candicans*) (Bottomley, 1909). These effects were ascribed to an increase in nitrogen (N) availability. However, the term PGPR was adopted almost 70 years later, at the Annual Meeting of the American Phytophatological Society (Kloepper and Schroth, 1979), where the mechanism of PGPR function was suggested to be via modification of the soil microflora. A year later, Kloepper et al. (1980) proposed that PGPR produce siderophores, which remove iron from the soil and reduce the growth of deleterious soil microorganisms. Although the definition of PGPR by Martínez-Rodríguez et al. (2014) included non-soil microorganisms that inhabit the aerial parts of the plants, this specific category of bacteria bring benefits to the plant through some positive root-microbe interactions (Zhou et al., 2015). They stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them. They also improve soil structure and bioremediating the polluted soils as they are capable of sequestering toxic heavy metal species and degrading xenobiotic compounds like pesticides. Indeed, the bacteria inhabiting around/in the plant roots are more efficient in transforming, mobilizing, solubilizing the nutrients than those from bulk soils (Hayat et al., 2010). Therefore, the rhizobacteria are the efficient and dominant driving forces in recycling the soil nutrients and thus are vital for soil fertility (Glick, 2012). Currently, the biological approaches for improving crop production are gaining strong status among agronomists and environmentalists following integrated plant nutrient management system. In this context, rigorous research are ongoing worldwide with greater momentum to explore a wide range of rhizobacteria possessing novel traits like heavy metal detoxifying potentials (Ma et al., 2011; Wani and Khan, 2010) pesticide degradation/tolerance (Ahemad and Khan, 2012), salinity tolerance (Mayak et al., 2004; Tank and Saraf, 2010), biological control of phytopathogens and insects (Joo et al., 2005; Hynes et al., 2008; Russo et al., 2008) along with the normal plant growth promoting properties such as, phytohormone (Ahemad and Khan, 2012; Tank and Saraf, 2010), 1-aminocyclopropane-1-carboxylate deaminase synthesis, hydrogen cyanate (HCN) and ammonia production, siderophore production (Jahanian et al., 2012; Tian et al., 2009), nitrogenase activity (Glick, 2012; Khan, 2005), phosphate solubilisation (Ahemad and Khan, 2012), trace element solubilisation (Kumar et al., 2012) etc. Hence, diverse symbiotic (*Rhizobium, Bradyrhizobium, Mesorhizobium*) and non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillum, Azomonas*), rhizobacteria are now being used worldwide as bio-inoculants to promote plant growth and

development under various stresses like salinity (Mayak et al., 2004), herbicides (Ahemad and Khan, 2011; Ahemad and Khan, 2010), insecticides (Ahemad and Khan, 2011), fungicides (Ahemad and Khan, 2012; Ahemad and Khan, 2011), heavy metals (Ma et al., 2011; Wani and Khan, 2010) etc.

1.3.3. Mechanisms of growth promotion of plants by PGPR

The beneficial effect of PGPR on plant growth and yield has been reported in several research works (Bergottini et al., 2015). PGPR employ various direct and indirect mechanisms to stimulate plant growth and development. They have direct mechanisms to facilitate uptake of nutrient, availability of nutrient through nitrogen fixation, solubilisation of complex organic nutrients, and production of phytohormones (Santoro et al., 2015; Grobelak et al., 2015; Gupta et al., 2015). They also indirectly promote plant growth by producing antibiotics, siderophores, HCN, hydrolytic enzymes etc (Fig. 1.4). Hence, the rhizobacteria constitute leading driving forces in revitalizing the soil health and are important for soil fertility (Babalola and Glick, 2012; Ahemad and Kibret, 2014).

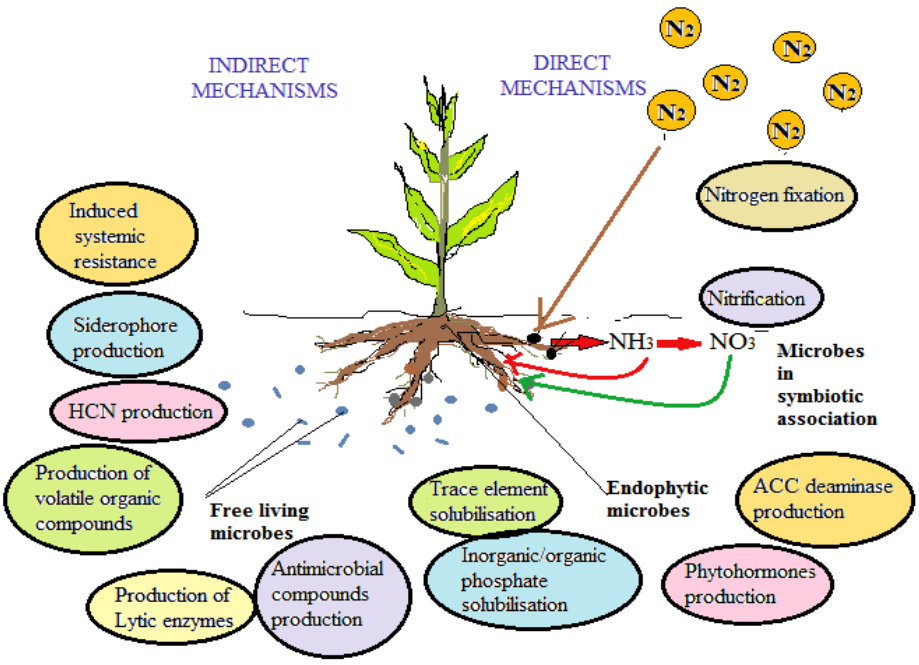

Fig 1.4. Direct and Indirect Mechanisms of action by PGPR on plant growth and development

1.3.3.1 Direct mechanisms of plant growth promotion by PGPR

The direct mechanisms of plant growth promotion by PGPR involve increased nutrient uptake and availability by nitrogen fixation, solubilisation of phosphate, potassium and trace elements, mineralization of organic compounds and phytohormone production.

1.3.3.1.1. Nitrogen fixation

Nitrogen (N) is a fundamental nutrient for plant growth and productivity. The atmospheric N_2 content is about 78%, however, it is unavailable to the growing plants directly. The atmospheric N_2 can be converted into plant utilizable forms by the biological process i.e. nitrogen fixation which involves conversion of atmospheric N_2 to ammonia by N_2-fixing microorganisms (Kim and Rees, 1994). N_2 fixation is a beneficial process which is an economical substitute to chemical fertilizers (Ladha et al., 1997). In the world, approximately two-thirds of the N_2 fixed are produced by biological processes whereas the remaining is synthesized industrially by the Haber-Bosch process (Rubio and Ludden, 2008). N_2-fixers are generally grouped as (a) symbiotic N_2-fixing bacteria and (b) non-symbiotic (free living and endophytes). Symbiotic N_2-fixers forms symbiotic association with leguminous (e.g. *Rhizobium* species) and non-leguminous (e.g. *Frankia*) plants (Ahemad and Khan, 2012; Zahran, 2001). The non-symbiotic N_2-fixing bacteria include cyanobacteria (*Anabaena, Nostoc*), *Azotobacter, Azospirillum, Azocarus, Gluconoacetobacter diazotrophicus* (Bhattacharyya and Jha, 2012); but they can add only a small amount of the fixed N_2compared to the total N requirement of the plant (Glick, 2012). Symbiotic N_2-fixing rhizobia under the family rhizobiaceae establish an infection thread that leads to their symbiotic relationship with the roots of leguminous plants. This symbiosis involves a complex interaction between the host and symbiotic microbes resulting in the formation of the nodules wherein the rhizobia colonize as intracellular symbionts (Fig. 1.5). Diazotrophs are the free living N_2 fixers in non-leguminous plants that are capable of establishing a non-obligate interaction with the host plants (Glick et al., 1999). N_2-fixing microorganisms carry out nitrogen fixation by the help of a complex enzyme system known as nitrogenase (Raymond et al., 2004, Kim and Rees, 1994). The complex structure of nitrogenase reported by Dean and Jacobson (1992) is a two-component metalloenzyme consisting of (i) dinitrogenase reductase which is an iron protein and (ii) dinitrogenase which has a metal cofactor. Dinitrogenase requires the electrons produced by dinitrogenase reductase to reduce N_2 to NH_3. Depending on the metal cofactor, three different N fixing systems have been identified (a) Mo-nitrogenase, (b) V-nitrogenase and (c) Fe-nitrogenase. Structurally, N_2-fixing system varies among different bacterial genera. Most nitrogen fixation is carried out by the activity of the molybdenum nitrogenase, which is found in all diazotrophs (Bishop and Jorerger, 1990). The genes for nitrogen fixation, called nif genes are found in both symbiotic and free living systems (Kim and Rees, 1994). Nitrogen fixation is a very energy demanding process which requires at least 16 mol of ATP for each mole of reduced nitrogen (NH_3).

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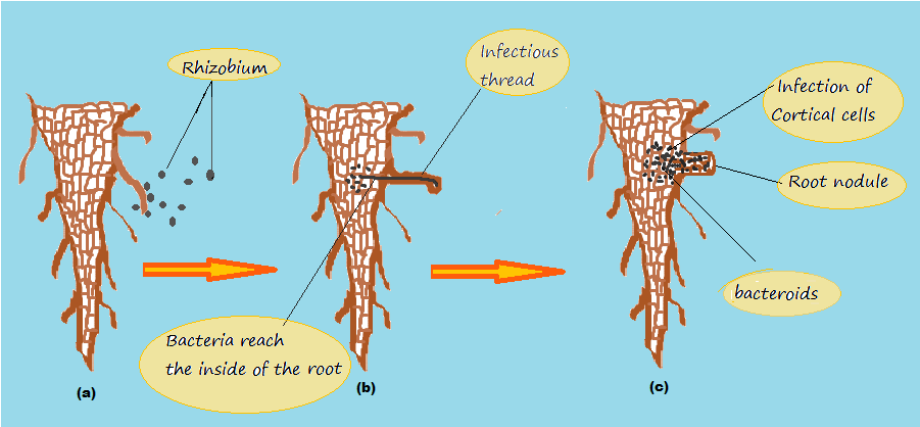


Fig 1.5. The nodulation process (a) Attachment of *Rhizobium* with root cells. (b) Secretion of nod factors by rhizobia causing root hair curling and formation of infectious thread through which the bacteria penetrate the cortical cells and (c) Formation of nodules

1.3.3.1.2. Phosphorous solubilisation

Phosphorus (P), the second important plant growth-limiting nutrient after N, is profusely available in soils in both organic and inorganic forms (Khan et al., 2009). In spite of soil being a large reservoir of P, the amount available to plants is generally low. The insoluble nature of P is an important factor for its unavailability to the plants. The plants absorb P only in two soluble forms, the monobasic (H_2PO_4) and the diabasic (HPO_4^{2-}) ions (Bhattacharyya and Jha, 2012). The insoluble P in soil is present either in the form of inorganic minerals, like apatite or as one of several organic forms, such assoil phytate (inositol phosphate), phosphomonesters and phosphotriesters (Glick, 2012). To overcome the deficiency of P in soils, there are frequent applications of phosphate fertilizers in crop fields. However, regular application of P-fertilizers is expensive and environmentally adverse. Plants can absorb only a fraction of applied P-fertilizers and the rest is rapidly converted into insoluble complexes in the soil (Mckenzie and Roberts, 1990). This problem has led to look for an ecologically safe and cost effective alternative for improving crop production in low P soils. In this context, organisms with phosphate solubilising activity, often termed as phosphate solubilizing microorganisms (PSMs), may provide the available forms of P to the plants and hence, can be a potent substitute to chemical P-fertilizers (Khan et al., 2006). Of the various PSMs inhabiting the rhizosphere, phosphate-solubilizing bacteria (PSB) are considered as promising biofertilizers since they can supply plants with P from sources otherwise poorly available by various mechanisms (Zaidi et al., 2009). The bacterial genera, *Azotobacter, Bacillus, Beijerinckia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* are reported to be the most significant PSB (Bhattacharyya and Jha, 2012). The

PSB secrete low molecular weight organic acids and carry out solubilisation of insoluble P (Zaidi et al., 2009). On the other hand, the mineralization of organic P occurs through the expression of a variety of different phosphatases by PSB, which catalyse the hydrolysis of phosphoric esters (Glick, 2012). The traits of phosphate solubilization and mineralization can exist altogether in the same bacterial strain (Tao et al., 2008). Though PSB are commonly found in most soil but their presence and performances are strictly affected by environmental factors especially under stress conditions (Ahemad and Khan, 2010; Ahemad and Khan, 2012). There are several reports wherein the effects of inoculation of PSB alone or in consortia are mentioned (Zaidi and Khan, 2005; Poonguzhali et al., 2008; Chen et al., 2008; Ahemad and Khan, 2012). Besides providing P to the plants, the PSB also augment the growth of plants by stimulating the efficiency of biological nitrogen fixers (BNF) and enhancing the availability of other trace elements by synthesizing important plant growth promoting substances (Suman et al., 2001; Ahmad et al., 2008; Zaidi et al., 2009)

1.3.3.1.3. Potassium solubilisation

After N and P, potassium (K) is the most important plant nutrient to play a key role in the plant growth, metabolism and development. K enhances plant resistance to diseases, pests, and abiotic stresses and activates over 80 different enzymes responsible for various plant processes such as energy metabolism, starch synthesis, nitrate reduction, photosynthesis and sugar degradation (Almeida et al., 2015; Cecílio Filho et al., 2015; Gallegos-Cedillo et al., 2016; Mumtaz et al., 2017; White and Karley, 2010; Yang et al., 2015). Depending on soil type, 90 to 98% of soil K is in mineral forms and most of them are not available for plant uptake (Sparks and Huang, 1985). PGPR could solubilize the insoluble K to soluble forms by various mechanisms including production of inorganic and organic acids, acidolysis, chelation and exchange reactions (Etesami et al., 2017). During K solubilization, the major mechanisms involved are the production of the organic acids, inorganic acids and protons (acidolysis mechanism) (Sheng et al., 2008; Maurya et al., 2014; Meena et al., 2014) which are able to convert the insoluble K (in the form of mica, muscovite, and biotite feldspar) to soluble forms that plants can uptake (Hu et al., 2006; Meena et al., 2014; Mo and Lian, 2011). In acidolysis mechanism, the released H^+ can help to dissolve the mineral K resulting in the slow release of readily available exchangeable K. Several organic acids such as oxalic, tartaric, gluconic, 2-ketogluconic, citric, malic, succinic, lactic, propionic, glycolic, malonic, and fumaric acid, have been reported to be produced and secreted by K solubilising bacteria, which are effective in releasing K from insoluble mineral-K (Krishnamurthy, 1989; Hu et al., 2006; Sheng and He, 2006; Liu et al., 2012; Prajapati et al., 2012; Prajapati et al., 2013; Saiyad et al., 2015).

1.3.3.1.4. Trace elements solubilisation

Zinc (Zn) is an essential micronutrient required for optimum plant growth. Plants can uptake Zn in the form of divalent cation (Zn^{2+}) (Kabata-Pendias and Pendias, 2001), however, only small proportion of total Zn is present as soluble form in the soil. Rest of the Zn is present in the form of insoluble complexes and minerals (Alloway, 2008) resulting in reduced availability to plants. Hence, the plant deficiency of Zn happens to be one of the most vital micronutrient deficiency. To overcome Zn deficiency, Zn fertilizers like $ZnSO_4$ (White and Broadly, 2005) or Zn-EDTA (Karak et al., 2005) are applied in agricultural lands. However, their usage has economic and environmental concern. After a week of application of Zn fertilizers, the chemical forms are converted into insoluble forms and remains in the soil (Rattan and Shukla, 1991). Hence, Zn solubilizing bacteria are potential alternatives for Zn supplementation that convert applied insoluble Zn to available forms which can be absorbed by the plants.

There are several PGPR that have been found to be effective Zn solubilizers. These bacteria colonize the rhizosphere and solubilize complex Zn compounds into simpler ones and thus making Zn available to the plants. Zn solubilizers have several mechanisms to solubilise Zn. One of such mechanisms is acidification process in which they produce organic acids to sequester the Zn^{2+} and decrease the pH of the nearby soil (Alexander, 1997). According to another report, the anions can also chelate Zn and enhance Zn solubility (Jones and Darrah, 1994). Zn solubilisation mechanisms involve the production of siderophores and proton (Saravanan et al., 2011). Further, oxidoreductive systems on cell membranes and chelated ligands are also responsible for Zn solubilisation (Chang et al., 2005; Kamran et al., 2017). Various bacterial genera showing enhanced Zn content and growth when applied to plants are *Pseudomonas, Rhizobium* strains (Deepak et al., 2013; Naz et al., 2016), *Bacillus aryabhattai* (Ramesh et al., 2014), *Bacillus* sp. (Hussain et al., 2016), and *Azospirillum*. Under laboratory condition, various bacterial strains viz. *Pseudomonas aeruginosa* (Fasim et al., 2002), *Gluconacetobacter diazotrophicus* (Saravanan et al., 2007), *Serratia liquefaciens, S. marcescens*, and *Bacillus thuringiensis* (Ullah et al., 2015), *Bacillus* sp., *Pseudomonas striata, Pseudomonas fluorescence, Burkholderia cenocepacia* (Pawar et al., 2015) were reported to show Zn solubilisation. These strains have been reported to increase Zn content of straw and grains in soybean and wheat (*Triticum aestivum*), enhancing food efficacy and dealing with deficiency of Zn. Vaid et al. (2014) reported the beneficial effect of inoculation of Zn solubilizing bacteria on rice plant growth and increased Zn nutrition (42.7%) of grains.

1.3.3.1.5. Phytohormones production

Phytohormones are the low molecular weight endogenous secondary metabolites which not only activate an effective defence response against both biotic and abiotic stresses but also act as regulators of growth, development and physiological processes of the plants.

PGPR produce several phytohormones such as auxins, cytokinins, giberellin, ethylene and abscissic acid. They add to the pre-existing hormone levels of the plant and have significant effects on overall plant growth and development. Microbial synthesis of the phytohormone auxin (indole-3-acetic acid, IAA) has been known for a long time. It is also known as a signalling molecule in some microorganisms (Bianco et al., 2006; Lui and Nester, 2006; Spaepen et al., 2007). PGPR produce auxins in order to affect host physiological processes for their own benefit (Shih-Yung, 2010). Patten and Glick (1996) reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins. IAA has been responsible in every aspect of plant growth and developmental processes as well as defence mechanisms. Generally, IAA is responsible for plant cell division, extension, and differentiation. It stimulates seed/tuber germination and also increases the rate of xylem and root development. Bacterial IAA provides the plant greater access to soil nutrients by increasing the root surface area and length. In addition as reported by Glick in 2012, rhizobacterial IAA loosens plant cell walls and helps in increasing the amount of root exudation providing an additional nutrients for growth of rhizobacteria. Therefore, rhizobacterial IAA is recognized as an important effector molecule in plant–microbe interactions (Spaepen and Vanderleyden, 2011). This variable range of functions is portrayed by the complexity of IAA biosynthesis, signalling and transport pathways (Santner et al., 2009). Tryptophan is an important amino acid that affects the synthesis of IAA as it is the main precursor for IAA biosynthesis (Zaidi et al., 2009). In one hand tryptophan being a precursor for IAA synthesis and on the other hand anthranilate which is a precursor for tryptophan, reduces IAA synthesis. By this mechanism, IAA biosynthesis is finely regulated by tryptophan with a negative feedback regulation on the enzyme anthranilate synthase and inhibiting anthranilate formation, and ultimately results in an indirect induction of IAA synthesis (Spaepen et al., 2007). Nevertheless, IAA production by most of the rhizobacteria increases when the culture media is supplemented with tryptophan (Spaepen and Vanderleyden, 2011). Cell division, differentiation and vascular bundle formation are essential for nodule formation and as IAA is involved in all these three processes. Hence, it can be concluded that IAA levels on plants are necessary for nodule formation (Spaepen et al., 2007; Glick, 2012). In one of the work by Camerini et al., 2008, inoculation of *Vicia hirsute* with *Rhizobium leguminosarum* bv. viciae wherein the IAA biosynthetic pathway had been introduced, produced root nodules containing 60-fold more IAA than nodules formed by the wild-type strain.

There are other phytohormones involving in the effects of rhizobacteria in plants. These include abscisic acid (ABA), cytokinins (CKs) and gibberellins (GAs). But these are not well studied. The excretion rate of root exudates containing amino acids along with other compounds as presented in table 1.1 is increasing the beneficial effects of CK levels in the plant to the PGPR (Kudoyarova et al., 2014). ABA is known for senescence or ageing of the plants. Although a very little amount is required for the growth as it helps in regulating

the stomata aperture and hence, transpiration rate and CO_2 uptake (Pospisilova, 2003). Some PGPR can reduce the levels of ABA and minimize the ageing process, thereby, increasing the plant growth (Belimov et al., 2014). Sometimes PGPR also increases the level of ABA content of the plant under water deficit conditions and resulting in reduction of water loss (Salomon et al., 2014). Furthermore, in 2014, Porcel et al. reported that when tomato plants deficient in ABA were inoculated with a *Bacillus megaterium* strain, there is a reduction in growth mainly due to an overproduction of ethylene. As a consequence, the positive effects of PGPR depend on the endogenous levels of ABA of the host plant.

1.3.3.1.6. Production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase

Ehtylene is also known as stress hormone other than its role in regulation of plant growth and development. The plant endogenous level of ethylene is considerably increased under stress conditions like drought, salinity, water logging, heavy metal contamination and pathogenicity which affects the overall performance of the plants in a negative way. As for example, the high level of ethylene in plants enhances defoliation and other cellular processes which can cause less crop yield (Saleem et al., 2007; Bhattacharyya and Jha, 2012). Plants treated with PGPR having the enzyme ACC deaminase show increased growth and development. ACC deaminase induces salt tolerance and reduces drought stress in plants by reducing the level of ethylene. (Nadeem et al., 2007; Zahir et al., 2008). Various bacterial genera have been identified with ACC deaminase production trait (activity). These are *Acinetobacter, Achromobacter, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Serratia* and *Rhizobium* etc. (Shaharoona et al., 2007; Nadeem et al., 2007; Zahir et al., 2008; Kang et al., 2010). The mechanism of action of ACC deaminase activity is breaking down ACC, an important precursor for ethylene production, into 2-oxobutanoate and NH_3 (Arshad et al., 2007). Effects of phytopathogens like viruses, bacteria and fungi are reduced by ACC deaminase producers. Not only that, these bacteria also relieve the stress from heavy metals, radiation, polyaromatic hydrocarbons, insect attack, high temperature, high light intensity and water-logging condition (Glick, 2012; Lugtenberg and Kamilova, 2009). The main effects of the seed or root inoculated/treated with ACC deaminase producing PGPR are root elongation, shoot growth, enhancement of root nodulation, mineral uptake and mycorrhizal colonization in many crop plants (Shaharoona et al., 2007; Nadeem et al., 2007; Glick, 2012).

1.3.3.2. Indirect mechanisms

In general, the main mechanisms of biocontrol activity of PGPR are competition for nutrients, niche exclusion, production of antifungal metabolites and siderophores and induced systemic resistance (Lugtenberg and Kamilova, 2009). Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, 2,4-diacetylphloroglucinol, tensin, pyrrolnitrin, pyoluteorin, phenazines and viscosinamide (Bhattacharyya and Jha, 2012). Interaction of some rhizobacteria with the plant roots can leads to plant resistance

against some phytopathogens like bacteria, fungi, and viruses. The phenomenon of gaining resistance in the plant is called induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009). Furthermore, ISR involving ethylene signalling and jasmonic acid within the plants can stimulate the defence responses of the host plant against a variety of phytopathogens (Glick, 2012). There are various microbial components, such as lipopolysaccharides (LPS), flagella, cyclic lipopeptides, homoserine lactones, 2,4-diacetylphloroglucinol, siderophores and volatiles like 2,3-butanediol and acetoin that can induce ISR (Lugtenberg and Kamilova, 2009).

1.3.3.2.1. Siderophore production

Iron is an important nutrient for almost all forms of life. Almost all microorganisms essentially require iron except lactobacilli (Neilands, 1995). Under aerobic condition, iron occurs mainly in the form of Fe^{3+}, which is likely to form insoluble hydroxides and oxyhydroxides making it unavailable to both plants and microorganisms (Rajkumar at al., 2010). PGPR obtain iron by secreting siderophores which are low molecular weight iron-chelating ligands having high affinity for iron produced under low iron stress (Verbon et al., 2017; Kumar et al., 2016). Production of siderophore has dual role, firstly, it helps in iron nutrition by acting as a solubilising agents for insoluble iron complexes and secondly, it inhibits phytopathogen. Siderophore producing PGPR compete for Fe^{3+} with the pathogens and thus create iron deficiency leading to the death of pathogen (Khurana and Sharma, 2000; Sharma and Kaur, 2010; Schiessl et al., 2017; Shaikh et al., 2016). Siderophores are usually water-soluble and can be divided into two types viz. extracellular siderophores and intracellular siderophores. Based on the ability of rhizobacteria for cross utilization of siderophore, there may be homologous siderophore (rhizobacteria proficient in using siderophores of the same genus) or heterologous siderophores (rhizobacteria with the ability to utilize siderophores produced by different genera) (Khan et al., 2009). Iron is present in the form of Fe^{3+} siderophore complex with both gram positive and gram negative bacterial membrane. The reduction of Fe^{3+} to Fe^{2+} lead to release of iron from the complex into the cell via a gating process which linked the bacterial inner and outer membranes. The reduction process either destroys the siderophore or may recycle it to capture more iron (Neilands, 1995; Rajkumar et al., 2010). Siderophores is not only form stable complex with Fe but also form complexes with other heavy metals like Al, Cd, Cu, Ga, In, Pb and Zn (Kiss and Farkas, 1998; Neubauer et al., 2000). There are different mechanisms by which plants assimilate iron from bacterial siderophores, for example chelation and release of iron, uptake of siderophore-Fe complexes directly, or by a ligand exchange reaction (Schmidt, 1999). There are numerous studies on plant growth promotion via siderophore-mediated Fe-uptake, obtained by inoculations of siderophore-producing rhizobacteria (Rajkumar et al., 2010). For example, a siderophore mediated iron transport system is reported in oat plants and rhizobacteria producing siderophores which delivers iron to the plant under iron-limited conditions (Crowley and Kraemer, 2007). Similarly, *Pseudomonas*

fluorescens C7 synthesised Fe-pyoverdine complex that was taken up by *Arabidopsis thaliana*. This led to an increase in plant iron content and improved plant growth (Vansuyt et al., 2007).

1.3.3.2.2. Production of Hydrogen cyanide (HCN)

HCN is produced during the initial stationary growth phase of bacteria. Though it does not play a role in growth, storage of energy or primary metabolism, but is generally play a significant ecological role and a selective advantage is conferred on the HCN producing strains (Vining, 1990). About 90% of *Pseudomonas* sp. have a common trait of HCN production (Ahmad et al., 2008). Cyanide is considered as one of the typical features of deleterious rhizobacteria as it is a phytotoxic agent capable of inhibiting the main enzymes involving in vital metabolic processes (Bakker and Schippers, 1987). However, its application as biocontrol agent is increasing (Devi et al. 2007). HCN produced in the rhizosphere of seedlings by selected rhizobacteria can be an effective mechanism of biocontrol. Therefore, HCN producing rhizobacteria have potential as ecofriendly way to control weeds and minimize adverse effects on the growth of the crop plants (Kremer and Souissi, 2001).

1.3.3.2.3. Production of protective enzymes

PGPR produce a number of metabolites that control phytopathogenic agents and promote plant growth (Meena et al., 2016). They produce enzymes such as chitinase, β-1,3-glucanase, and ACC-deaminase which are generally involved in degradation of cell walls and neutralizing pathogens (Goswami et al., 2016). Almost all fungal cell walls are constituted of β-1,4-N-acetyl-glucosamine and chitin and therefore, β-1,3-glucanase and chitinase-producing bacteria control fungal growth. *Fusarium oxysporum* and *Fusarium udum* causing fusarium wilt can be inhibited by *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 which can produce beta-glucanases and chitinase (Ramadan et al., 2016). Islam et al. (2016) reported that *Phytophthora capsici* and *Rhizoctonia solani*, the most disastrous crop pathogens in the world, are also inhibited by PGPR.

1.3.3.2.4. Disease resistance antibiosis

For sustainable agriculture use of microbes producing antagonistic compounds against phytopathogens has been recommended as an alternative to chemical pesticides. PGPR, like *Bacillus* sp. and *Pseudomonas* sp., capable of producing antibiotics play a significant role in inhibiting pathogenic microorganisms. Over the past 20 years, antibiotic synthesis by PGPR against several phytopathogens has become one of the most studied and effective mechanism of biocontrol (Ulloa-Ogaz et al., 2015). It is reported that most of the species of *Pseudomonas* genera produce a wide variety of (a) antifungal antibiotics viz. phenazines, phenazine-1-carboxamide, phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol,

pyrrolnitrin, rhamnolipids, pyoluteorin, cepaciamide A, viscosinamide, oomycin A, ecomycins, butyrolactones, N-butylbenzenesulfonamide, pyocyanin (b) bacterial antibiotics like azomycin and pseudomonic acid (c) antitumor antibiotics (e.g. cepafungins and FR901463) and (d) antiviral antibiotics (Karalicine) (Ramadan et al., 2016). Further, the antibiotics can be grouped into volatile compounds including aldehydes, alcohols, ketones, hydrogencyanide, sulfides, etc., and non-volatile compounds, including aminopolyols, cycliclipopeptides, phenylpyrrole, polyketides, heterocyclic nitrogenous compounds etc. (Fouzia et al., 2015). *Bacillus* species are also capable of producing a wide variety of antifungal and antibacterial lipopeptide antibiotics, like surfactin, iturins, and bacillomycin (Wang et al., 2015).

1.3.3.2.5. Induced systemic resistance

Induced systemic resistance (ISR) is the term to define the physiological state of improved defensive capacity evoked in plants in response to a particular environmental stimulus. Induced systemic resistance in many plants is raised by PGPR against several environmental stresses (Prathap and Ranjitha, 2015) especially during phytopathogen invasion. During pathogenic invasion, signal molecules are produced and a defense mechanism is activated via the vascular system resulting in the activation of a vast number of defense enzymes viz. β-1,3-glucanase, chitinase, peroxidase, phenylalanine ammonia lyase, polyphenol oxidase, lipoxygenase, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidise (APX) along with some proteinase inhibitors. Kamal et al. (2014) reported that ISR functions in a broadway and is not specific against a particular pathogen. ISR involves the signalling of ethylene hormone within the plant that induces the defense responses of a host plant against a variety of phytopathogens. A variety of individual bacterial components also induce ISR, such as cyclic lipopeptides, homoserine lactones, lipopolysaccharides, siderophores, 2,4-diacetylphloroglucinol, and volatiles, like acetoin and 2, 3-butanediol (Berendsen et al., 2015).

Table 1.2.
Effect of PGPR on various plants

Organisms	IAA production	Phosphate solubilization	Phytase production	Application on crops	Result on crops	References
Bacillus Amyloliquefaciens FZB45	D.A	D.A	0.37 U mL^{-1}	*Zea mays* cv. Elita	Shoot and root wt increased, root length increased, support plant growth at low phosphate cond.	Idriss et al., 2002
Pseudomonas	D.A	24.7-44 mg $100ml^{-1}$	D.A	Chinese cabbage	D.A	Poonguzhali et al., 2008
Bacillus	17 µg mL^{-1}	23 mg mL^{-1} TCP 20 mg mL^{-1} DCP 19 mg mL^{-1} ZP	D.A	D.A	D.A	Pankaj et al., 2012
B.subtilis	7 µg mL^{-1}	D.A	D.A	D.A	D.A	Mohite, 2013
Lactobacillus casei	2 µg mL^{-1}	D.A	D.A	D.A	D.A	Mohite, 2013
B.cereus	6 µg mL^{-1}	D.A	D.A	D.A	D.A	Mohite, 2013
Pseudomonas fluroscens	15.38±0.537	D.A	D.A	Onion	D.A	Reetha et al. 2014
Bacillus subtilis	12.67±0.325	D.A	D.A	onion	D.A	Reetha et al., 2014
Bacillus	50.3 µg mL^{-1}	9.77 mg 100 mL^{-1}	D.A	Mungbean	D.A	Kaur and Sharma, 2013
Pseudomonas	55.6 µg mL^{-1}	9.14 mg 100 mL^{-1}	D.A	Mungbean	D.A	Kaur and Sharma, 2013
Azotobacter	52.6 µg mL^{-1}	8.98 mg 100 mL^{-1}	D.A	Mungbean	D.A	Kaur and Sharma, 2013
Advenella sp. PB6	25 µg mL^{-1}	~620 µg mL^{-1}	0.174 U mL^{-1}	*Brassica*	Increased root length	Singh et al., 2014
*Advenella*sp. PB10	35 µg mL^{-1}	~650 µg mL^{-1}	0.161	*Brassica*	Increased shoot length	Singh et al., 2014
*Cellulosimicrobim*s p. Pb9	10 µg mL^{-1}	746 µg mL^{-1}	0.129 U mL^{-1}	*Brassica*		Singh et al., 2014
Streptomyces cellulosae mhcr0816	136.5 mg L^{-1}	1916 mg L^{-1}	0.68 U mL^{-1}	*Triticum aestivum*	improved plant growth, biomass (33 %) and mineral (Fe, Mn, P) content	Jog et al., 2014
Pseudomonas. aeroginosa	3.6 mg L^{-1}	D.A	D.A	*Zea mays* L.	D.A	Kumar, 2015
Azotobacter chrooccum	3 mg L^{-1}	D.A	D.A	*Zea mays* L.	D.A	Kumar, 2015
Azospirillum brasilense	2.3 mg L^{-1}	D.A	D.A	*Zea mays* L.	D.A	Kumar, 2015
Streptomyces sp.	1.9 mg L^{-1}	D.A	D.A	*Zea mays* L.	D.A	Kumar, 2015

Enterobacter cloacae	8.98±0.46 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
Stenotrophomonasmaltophilia	3.6 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
Pseudomonas veronii	4.5 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
P. validus	7.0 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
Baciliussubtilis	7.2 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
Bacillus licheniformis	7.2 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
Bacillusamyloliquefaciens	5.5 mg L^{-1}	D.A	D.A	D.A	D.A	Haiyambo, 2015
Pseudomonas fluorescens L228	D.A	1312 mg L^{-1}	D.A	*Pisum sativum*	Improved plant growth	Oteino et al., 2015
Aneurinibacillusaneurinilyticus CKMV1	8.1 µg mL-1	230 mg/l	D.A	tomato	Shoot and root length increased, Dry wt of shoot and root increased	Chauhan et al., 2017
MRS34	28.41 µg mL^{-1}	23.9 µg mL^{-1}	D.A	*Zea mays*	D.A	Manzoor et al., 2016
Enterobacter cloacae	12.125 µg mL^{-1}	D.A	D.A	*Triticum aestivum*	contributed to increase lengths of roots and shoots	Kamran et al., 2017
Bacillus sp.	D.A	D.A	60 µmol L^{-1}	*Zea mays* cv Jidan3	Increased plant height and biomass	Liu et al., 2018
Pseudomonas koreensis MS16	25.6±1.40 µg mL^{-1}	0.5 mg mL^{-1}	D.A	*Triticum aestivum*	Increased plant biomass and grain yield	Suleman et al., 2018
Enterobacter cloacae MS32	28.1±1.23 µg mL^{-1}	0.270 mg mL^{-1}	D.A	*Triticum aestivum*	Increased plant biomass and grain yield	Suleman et al., 2018

D.A= Data absent

1.3.3.2.6. Production of volatile organic compounds

Volatile Organic Compounds (VOCs) produced by rhizobacteria are low molecular weight (MW) compounds with < 300 g mol^{-1} MW and high vapour pressure. These include aldehydes, alcohol, ketones, acids, terpenes and hydrocarobons (Bhattacharyya et al., 2017; Fincheira and Quiroz, 2018). There is a direct relation between VOCs with ISR (Shafi et al., 2017). There are other types of VOCs like indole, 2,3-butanediol, 3-hydroxy-2-butanone (acetoin), cyclohexane, 2-(benzyloxy) ethanamine, benzene, benzene(1-methylnonadecyl), methyl, 1-chlorooctadecane, decane, 1-(N-phenylcarbamyl)- 2-morpholinocyclohexene, dodecane, 2,6,10-trimethyl dotriacontane, 11-decyldocosane, tetradecane, mixture of volatile compounds including Caryophyllene, which can promote the growth of plants, although the identity and quantity of the VOCs emitted vary among species (Ryu et al., 2003, Minerdi et al., 2011; Bailly and Weisskopf, 2012; Kanchiswamy et al., 2015). However, in the absence of pathogens they can also promote plant growth and confer tolerance against abiotic stresses (Bhattacharyya et al., 2015). In 2003, Ryu et al. confirmed the effect of plant promotion by the VOCs by using bacterial mutants which are unable to produce these VOCs or by the application of the pure compounds. VOCs like 2-pentylfuran was shown to increase fresh weight of *Arabidopsis. thaliana*, with an optimum dose of 10 g (Zou et al.,2010). Bailly and Weisskopf (2012) proposed that most of the studies dealing with the effect of VOCs on plant have been carried out using *A. thaliana* as a target for bacterial volatile compounds. Some of the compounds have been proven to promote growth but the actual reason is not clear whether it is due to a specific VOC. Further, many VOCs has inhibitory effects on the growth of plants at high dose and even some are toxic also (Bailly and Weisskopf, 2012). Therefore, it has become important to elucidate the signalling cascades and subsequent metabolic changes that are triggered in the plant by VOCs or VOCs producing PGPR (Ahemad and Kibret, 2014). Several bacterial species from diverse genera producing VOCs include *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* that enhance plant growth. In 2016, Santoro et al. mentioned that 2, 3-Butanediol and acetoin produced by *Bacillus* sp. are most effective for inhibiting fungal growth and promoting growth of the plants. It has been reported by Sharifi and Ryu, (2016) that bacterial VOCs are important determinants for eliciting plant ISR.

1.3.4. Nitrogen Use Efficiency of plants

The deleterious effect of excess N in the environment is not an unknown fact. Rather there should be improvement in the Nitrogen Use efficiency (NUE) of the plants which has become a challenge nowadays. There are several interacting genetic and environmental factors and so NUE is inherently a complex entity. The definition of NUE itself is also complex, and the term can mean different things in different contexts, including N use efficiency (NUE), N uptake efficiency (NUpE), N utilization (assimilation) efficiency (NUtE). NUtE is defined as total seed yield relative to total shoot N content (Moll et al.,

1982) and is affected by several physiological factors, including N uptake, metabolism, allocation, and remobilization (Girondé et al., 2015). Apparent N recovery rate (ANR), agronomy efficiency of fertilizer N (AE), N physiological use efficiency (NpUE), N transport efficiency (NTE), and N remobilization efficiency (NRE). Table 1.3 shows the definitions of all the terms. Mainly two plant physiological components: NUpE and NUtE that contribute to plant NUE.

Table 1.3.
Definitions of various terms related to plant's efficiency of N use

Terms	Meanings and formula
N use efficiency (NUE)	The total biomass or grain yield produced per unit of applied fertilizer N; it is an integration of NUpE and NUtE NUE= NUpE+NUtE
N uptake efficiency (NUpE)	The capacity of plant roots to acquire N from the soil (commonly referred to as the percentage of fertilizer N acquired by plant) NUpE= % of fertilizer N acquired by plant
N utilization (assimilation) efficiency (NUtE)	The fraction of plant acquired N to be converted to total plant biomass or grain yield NUtE=total plant biomass or grain yield
Apparent N recovery rate (ANR)	The ratio of net increased total N uptake by the plant with and without N fertilization to total amount of fertilizer N ANR= (N uptake by plant with N fertilization)-(N uptake w/o N fertilization) / Total amount of fertilizer N
Agronomy efficiency of fertilizer N (AE)	The ratio of net increased grain weight of the plant with and without N fertilization to total amount of fertilizer N AE= (Grain weight of the plant with fertilization)-(Grain weight of the plant w/o N fertilization) / Total amount of fertilizer N
N physiological use efficiency (NpUE)	The ratio of net increased grain weight to net increased N uptake with and without application of fertilizer N NpUE=Grain weight of the plant with fertilization -Grain weight of the plant w/o N fertilization / (N uptake by plant with N fertilization)- (N uptake w/o N fertilization) =ANR/AE
N transport efficiency (NTE)	The ratio of total N transported into the above ground parts to total N in the whole plant NTE = Total N transported into the above ground parts / Total N in the whole plant
N remobilization efficiency (NRE)	The ratio of N remobilization from source or senescent leaves to that of sink leaves or developing grains (seeds) NRE= N remobilization from source or senescent leaves / N of sink leaves or seeds

1.3.5. Plant nitrogen acquisition

Nitrate is the chief form of inorganic N in aerobic soil whereas ammonium being the major form in flooded field or acidic soils. The redox potential of the soil is influenced by the release of oxygen from root, which in turn accomplish the interconversion of soil N forms, including those derived from fertilizer. The concentration of nitrate and ammonium in the soil is very dynamic and heterogenous. The range varies from 100 µM to 10 mM. To adapt with the fluctuative situation, plant roots have uptake systems with different affinities for nitrate and ammonium. The uptake system consists of a number of membrane proteins that participate in the acquisition of N involving nitrate uptake, compartmentation, translocation, and remobilization by the plants (Dechorgnat, 2011). Each high and low-affinity nitrate transport system is composed of constitutive and nitrate-inducible components (Forde, 2000). The acquisition of N by the roots is affected by the root architecture, concentration of nitrate and ammonium in the soil, transporters systems as well as temperature fluctuations.

1.3.6. Nitrogen assimilation

Acquisition of N is followed by the assimilation process. In some plants, a very small amount of nitrate is assilmilated into the roots but larger portion of the absorbed N is assimilated in the shoot parts of the plants. Nitrate assimilation is carried out by the cytosolic nitrate reductase that catalyses the reduction of nitrate to nitrite which is further reduced to ammonia by nitrite reductase in the plastids. The ammonium derived either from nitrate or directly absorbed through ammonium transporters (AMTs) is further assimilated into amino acids via the GS/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle (Pinheiro and Chaves, 2011). The predominant GS/GOGAT isoenzymes are chloroplastic GS2 and Fd-GOGAT and cytosolic GS1 and NADH-GOGAT.

1.3.7. Nitrogen Transportation and Remobilization

The transportation of nitrate to different parts of the plants is conducted by the nitrate (NO_3^-) transporters systems. In *Arabidopsis,* there are two closely related low affinity nitrate transporters (NRT 1s) viz. AtNRT1.5 and AtNRT1.8 which are responsible for the loading and unloading of the nitrate into the root stele or from the shoot. The loading of nitrate into the root phloem is facilitated by another type of transporter AtNRT1.9 present in the companion cells which enhances downward transport of nitrate in roots. During vegetative stage of the plants, N is excessively present in the leaves. As the plant matures and proceeds towards senescence this N is remobilized to the developing seeds. A less intensive study has been done on the uptake of ammonium (NH_4^+) than nitrate uptake. Many species of plants using nitrate transporters systems can also have an efficient system for absorption of ammonium ions. This transporter systems are known as AMT (AtAMT in *Arabidopsis*) and express constitutively at high level of NH_4^+ (Yuan et al., 2007).

AMT can transport N either in the form hydrophobic NH_3 or charged ammonium. Glass et al. in 2002 reported that AMT1 family of high-affinity NH_4^+ transporters contains five members. Out of which AtAMT1.1, AtAMT1.2, and AtAMT1.3 have been extensively studied. Plant species and the type of transporters are important factors upon which the locations of the expression of transporter genes take place. According to various studies, in roots AtAMT1.2, AtAMT1.3 and AtAMT1.5 genes are expressed while only AtATM1.1 is expressed in leaf and root tissues (Forde and Clarkson, 1999; Glass et al., 2002; Khademi et al., 2004). Also, the AtAMT transporters are found in different types of root tissues. According to Ludewlg et al. (2007), AtAMT1.1, AtAMT1.3, and AtAMT1.5 are localized in the cell membrane of rhizodermis cells, while AtAMT1.2 is present in the plasma membrane of cortical and endodermal cells.

According to Calvo (2019), AMT1.1, AMT1.2, AMT1.3, and AMT1.5 genes showed high transcript levels in *A. thaliana* plants treated with three different PGPR mixtures, which demonstrates that PGPR affect ammonium transport in different root tissues. The relative contribution of these transporters to nitrate uptake depends on the developmental stage of the root and the N status of the plant (Wang et al., 2012).

1.3.8. Role of PGPR in Nitrogen status of Plants

PGPR play significant role in promoting the nitrogen status in plants. In 1906, J.F. Breazeale demonstrated that wheat plants that were starved for nitrogen for the first 15 days after germination subsequently showed much higher capacities for absorbing nitrate than plants that had received sufficient N (Breazeale, 1906). Glutamine, the product of the first step in the pathway of N assimilation in bacteria and fungi as well as in plants (Lea and Miflin, 2018), is the organic form of N that has been generally considered to be a key effector in the sensing of the intracellular N status in many organisms. In microbes like *Aspergillus nidulans* and other filamentous fungi, when glutamine levels are high, pathways that are responsible for assimilating N sources such as nitrate are down-regulated through a process named 'nitrogen metabolite repression' (Crawford and Arst, 1993, Siverio, 2002, Pfannmüller et al., 2017). Plant growth is enhanced by PGP under the effect of multigenic processes, including nitrate (NO_3^-) and ammonium (NH_4^+) uptake genes, which could potentially describe the upgrade in plant nutrition and plant growth. In 2000, Bertrand et al. showed that a strain of *Achromobacter* spp. improved the rate of nitrate (NO_3^-) uptake of *Brassica napus* roots.

1.3.9. Response Surface Methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modelling and analysis in applications where a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery and Runger, 2011). RSM is proved to a suitable mathematical and statistical

tool. The main aim of this approach is to optimize the response with minimum number of experiment. The response is called the dependable variables whereas the parameters that affect the responses are called the independent variable. The interaction effect between the independent variables are being analysed under optimal operating condition for a model or system to optimize the output or response variables (Azargohar et al., 2005). The two main experimental design which are used in RSM are Box-Behnken designs (BBD) and central composite design (CCD) (Bezerra et al., 2008; Zolgharnein et al., 2013). In very recently Central composite rotatable design (CCRD) with axial points and face central composite design (FCCD) has been used in RSM to give the model much flexibility and better optimize the response (Wang et al., 2018).The experimental data were analysed using the several statistical equation viz. Linear, Quadratic, Cubic or 2FI (two factor interaction) to fit in and generation in the model. Models are being generated with constant coefficient term such as liner coefficients for input variables (A, B, C etc.), interaction coefficient term (AB, AC, BC), quadratic coefficient term (A^2, B^2 and C^2). Coefficient of determinant (R^2) close to 1, adjusted R2>0.8, adequate precision (>4), model level of significance ($p<0.05$) and model lack of fit ($p>0.05$) are desirable to validate the model (Saha and Ghosh, 2014). Once the model is validated it can be used to predict the values of input variables in which optimum response will be achievable. Therefore, with minimum number of experiment in RSM as compared to quite large number of experiment in conventional approach, RSM help to reduce disadvantages associated with conventional method.

CHAPTER TWO

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF POTENT PLANT GROWTH PROMOTING RHIZOBACTERIAL (PGPR) STRAINS

2.1. Introduction

Nitrogen (N) is a limiting factor for agricultural crop productivity. Among the macronutrients required by plants, N is required in the greatest quantities for biosynthesis of amino acids, proteins, DNA, RNA, phytohormones and other secondary metabolites. Therefore, N-fertilizers, mainly applied in the form of ammonium (NH_4^+) and nitrate (NO_3^-), play significant role in increasing the productivity of major food crops throughout the world (Chardon et al., 2012; Lassaletta et al., 2014). They have intense effect on the growth and development of plants including growth of roots (Krouk et al., 2010; Lima et al., 2010) and shoots (Hirel et al., 2007; Tschoep et al., 2009). Although the production and application of chemical N-fertilizers have resulted in increased food yield globally and combating world hunger during the past 50 years, their increased use in intensive agriculture has created serious environmental hazards and health issues (Zhang et al., 2012). As plants are unable to use fertilizers efficiently, excess N compounds released from agricultural land create threat to the quality of air, water, and soil. Release of nitrous oxide and other reactive N gases into atmosphere and increase in soil leaching into drainage water, can cause soil acidification causing loss of plant productivity, eutrophication of water bodies leading to death to aquatic life and air pollution (Ottman and Pope, 2000; Diaz and Rosenberg, 2008; Xu et al., 2012; Erisman et al., 2013). Moreover, NO_3^- that accumulates in edible vegetative organs has been considered as source of potential threat to human health. Excessive application of N-fertilizers also impairs soil productivity leading to reduced N use efficiency (NUE) and stagnation of crop yield (Lawlor, 2002; Yousaf et al., 2016). Therefore, there is increasing concern regarding sustainability of technology to provide long-term food security to a growing population. The biggest challenge of 21st century is to continue agricultural productivity in a way that reduces the application of N-fertilizers (Hera, 1995; Xu et al., 2012; Backer et al., 2018).

Researchers all over the world are taking keen interest in achieving better use efficiency of fertilizers with the aid of plant growth promoting rhizobacteria (PGPR) as supplements to fertilizers (Rosier et al., 2018; Xu et al., 2019). PGPR are the soil bacteria inhabiting around/on the root surface that promote plant productivity. They affect plant growth either directly through ability to supply N, phosphorous, potassium and other essential minerals or indirectly through interfering with attacking pathogens (Kloepper

and Schroth, 1981; Olanrewaju et al., 2017). They can be a promising component for integrated solutions to environmental problems because of their capacity to increase seed germination rate, root and shoot growth, chlorophyll content, nutrient uptake, protein and carbohydrate contents, hydraulic activity, tolerance to biotic and abiotic stress, crop yield and delayed ageing or senescence (Mahaffee and Kloepper, 1997; Backer et al., 2018). PGPR play significant role in N cycling and plant utilization of fertilizer N in the plant-soil system (Ames et al., 1983; Adesemoye et al., 2009). Atmospheric nitrogen (N_2) is relatively inert and it does not easily react with other chemicals to form new compounds. N_2 is converted into NH_4^+ by biological nitrogen fixation. Plant N uptake through symbiotic N fixation (Elsheikh and Elzidany, 1997) and non-legume biological fixation/non-associative uptake have been reported (Kennedy et al., 1997; Dobbelaere et al., 2001). The NH_4^+ formed is further oxidized to nitrite (NO_2^-) and NO_3^- by the process of nitrification, which is carried out by nitrifying bacteria, like *Nitrosomonas*, *Nitrosococcus*, *Nitrobacter*, *Nitrococcus* etc. Nitrification is important in agricultural system where fertilizer is often applied as NH_4^+. Conversion of NH_4^+ to NO_3^- increases N absorption as because NO_3^- is more water-soluble than NH_4^+ (Li et al., 2008).

The NO_3^- absorbed by the plant root is assimilated into NH_4^+ via nitrate reduction pathways, whereas NH_4^+ either absorbed directly through root or produced by nitrate reduction is further assimilated by ammonia assimilation pathway involving Glutamine synthetase (GS)/glutamine oxo-glutarate amino transferase (GOGAT). GS catalyses the ATP-dependent condensation of NH_4^+ with glutamate to yield glutamine, whereas GOGAT catalyses the reaction of glutamine with 2-oxoglutarate to yield two molecules of glutamate. The net outcome of the GS/GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids and nitrogenous compounds (Forde and Lea, 2007; Bernard and Habash, 2009). Plant growth and ultimately, biomass accumulation are critically determined by coordination of N metabolism with carbon metabolism and their optimal functioning (Krapp and Truong, 2005). The reducing equivalents, primarily NADPH produced in photosynthesis are used in the synthesis of C and N assimilates of many different types, particularly carbohydrates and amino acids. These fuel the synthesis of biochemical components and ultimately structure of the whole plant (Krapp and Truong, 2005). Moreover, NO_3^- and other forms of N can also act as signals that regulate the expression of hundreds of genes involved in plant metabolism, physiology, growth and development (Vidal et al., 2010).

Present investigation aims at isolation of PGPR from rhizosphere, their qualitative and quantitative screening for N-fixation and other plant growth promoting (PGP) traits, their identification by using morphological, biochemical methods and finally, selection of potent PGPR for plant application on the basis of principal component analysis (PCA).

2.2. Materials and Methods

2.2.1. Chemical and reagents

All chemicals were from Sigma-Aldrich, USA; Merck, Germany; SRL, India; Promega, USA and were of analytical grade. Microbiological media were purchased from HiMedia Laboratory, India.

2.2.2. Collection of rhizospheric soil

During winters chickpea rhizospheric soil sample was collected from the agricultural field (26°42' 30.88" N longitude and 88°20'62 52.97" E latitude) near University of North Bengal, Siliguri, West Bengal, India. Approximately 10 g of rhizospheric soil sample was collected and kept in sterilized zipped bag. The bags were immediately taken to the laboratory of Department of Biotechnology, University of North Bengal.

2.2.3. Isolation of microorganism from soil

Isolation of rhizospheric bacteria was done by using standard serial dilution and spread plate techniques. 1g soil was suspended in 9 mL of standard 0.85 % saline and vortexed for 10 min to get a uniform suspension. Soil suspension was serial diluted (10^{-1} to 10^{-8}) and 0.1 mL of the serially diluted suspensions were spread plated on nutrient agar and incubated at 37°C for 24 h. The colonies with distinct morphology were observed carefully and isolated. Further the colonies were purified using quadrant streak method on nutrient agar plate and maintained in nutrient agar slant for use. Glycerol stock of the isolated microorganisms was also made and stored at -20°C for further use.

2.2.4. Screening of bacterial isolates for plant growth promoting (PGP) traits

All the isolated strains were tested for their plant growth promoting (PGP) attributes qualitatively and quantitatively, like N-fixation, ammonia production, phosphate solubilisation, zinc solubilisation, synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase.

2.2.4.1. Nitrogen fixation and ammonia production

Bacterial culture grown overnight on nutrient broth (NB) was inoculated into the 100 mL of Asbhy's N-free liquid (ANFL) medium containing (g L^{-1}) mannitol, 15; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.2; $CaCl_2$, 0.2; $FeCl_3$,0.05 and 1 drop of 10% Na_2MoO_4 (w/v) and incubated at 37 ºC for 24 h. Thereafter, the culture was streaked on Asbhy's N-free agar (ANFA) plates (ANFL + 2% w/v agar) and incubated at 37 ºC for 24h. Bacterial strains showing positive growth on the ANFA plates were further checked for quantitative production of ammonia following the method of Goswami et al. (2014). Bacterial culture grown in ANFL medium for 24 h at 37 ºC was centrifuged at 8000 rpm for 10 min at 4 ºC to obtain the clear supernatant. To 0.2 mL of culture supernatant 1 mL Nessler's reagent (Himedia) and 7.3 mL of deionized water were added. The reaction mixture was shaken well and incubated at room temperature (RT) for 15 min. Development of brown to yellow colour is indicative of ammonia production. Ammonia production was estimated spectrophotometrically at 450 nm using the standard curve prepared with 1-10 µg mL^{-1} ammonium sulphate (Fig.2.1).

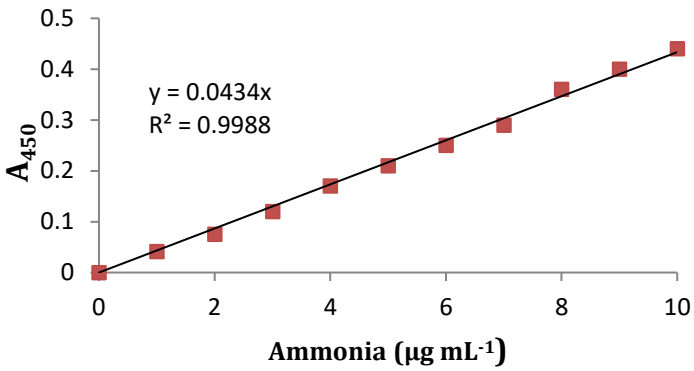


Fig 2.1. Standard curve of Ammonia

2.2.4.2. Screening of free living diazotrophs (Non-symbiotic N fixation)

Screening of free living diazotrophs was done by growing the strains in Jacob's N-free broth (JNFb) medium). Growth capacity of the isolate in N-free medium was detected by the method of Ribeiro and Cardoso (2012). Bacterial isolate was inoculated in screw-cap tube containing 10 mL JNFb liquid medium of the following composition (in g L^{-1}): DL-malic acid, 5; K_2HPO_4, 0.13; $MgSO_4.7H_2O$, 0.25; NaCl, 1.20; $CaCl_2.2H_2O$, 0.25; Na_2SO_4, 2.4; $NaHCO_3$, 0.22; Na_2CO_3, 0.09; K_2SO_4 0.17; agar 1.75; minor element solution 2 mL L^{-1}; Fe-EDTA (1.64%) 4 mL L^{-1}; Vitamin solution 1 mL L^{-1}; pH 7 [Minor element solution consisted of (in g L^{-1}) $CuSO_4.5H_2O$, 0.4; $ZnSO_4.7H_2O$, 0.12; H_2BO_3, 1.4; $Na_2MoO_4.2H_2O$, 1; $MnSO_4.H_2O$, 1.5. Vitamin solution contained (in mg L^{-1}) biotin, 100 g; pyridoxal HCl, 200] and then grown at 37 ºC for 24 h. From there, 1% (v/v) culture was transferred to JNF semi-solid culture medium and incubated at 37 ºC for 7 days. Free living diazotroph was identified by the formation of pellicle in JNF semi-solid media.

2.2.4.3. Solubilisation of inorganic phosphate (Pi)

Phosphate solubilisation property of bacterial isolates was determined by the method of Katznelson and Bose (1959). Bacterial culture was grown on NB for overnight at 37 ºC and then 0.1 mL of the culture was transferred to 100 mL of Pikovskaya broth (PKB) containing (g L^{-1}) glucose, 10; dicalcium (DCP) or tricalcium phosphate (TCP), 5; $(NH_4)_2SO_4$ 0.5; NaCl, 0.2; $MgSO_4$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4.H_2O$, 0.0001; $FeSO_4.7H_2O$ 0.0001, pH 7 and then grown at 37 ºC for 48 h. The culture obtained was point inoculated on Pikovskaya agar (PKA=PKB + Agar) plate containing DCP or TCP as insoluble Pi source and incubated at 37 ºC for around 7 days and then observed for appearance of clear zone around the colonies. Phosphate solubilisation index (PSI) was calculated (Vazquez et al., 2000) from the following equation:

$$PSI = \frac{\text{Diameter of phosphate solubilization zone (mm)}}{\text{Growth diameter of spot inoculant (mm)}} \quad \text{(Eq 1)}$$

The quantitative phosphate liberation capacity of the isolates was evaluated using the Vanadomolybdophosphoric acid method of Barton (1948). For this, isolate showing significant PSI was grown in 100 mL of PKB containing DCP, for 48 h at 37 ºC. The

culture broth was then centrifuged at 10,000 rpm for 10 min at 4°C to obtain the cell free extract (CFE). To 1 mL of CFE 0.5mL of Vanado-molybdate reagent [2.5 g ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$) dissolved in 30 mL dH_2O (Solution A) and 0.125 g ammonium metavanadate (NH_4VO_3) dissolved in 30 mL dH_2O by heating and 33 mL of concentrated HCl added after cooling (solution-B), and finally, 30 mL of solution-A mixed with 63 mL of solution-B and the final volume of the mixture made 100 mL with dH_2O] was added. The resulting reaction mixture was incubated for 10 min and thereafter, absorbance was recorded at 400 nm using spectrophotometer. The amount of solubilised phosphate was estimated using standard curve of KH_2PO_4 (10-100 µg mL^{-1}) as reference (Fig 2.2).

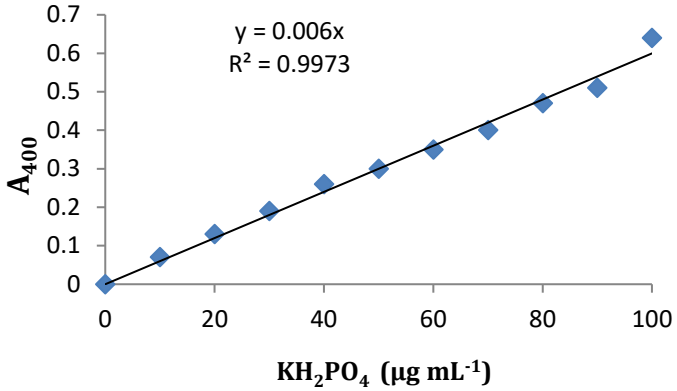


Fig 2.2. Standard curve of KH_2PO_4

2.2.4.4. Solubilisation of organic phosphate (OP)

Screening of bacterial isolate for organic phosphate (sodium phytate) solubilisation capacity was based on the production of phytase that hydrolyze phytate to release inorganic phosphate (Pi). Isolated organisms were grown in phytate screening media (PSM) containing (g L^{-1}): glucose, 20; KCl, 0.5; $CaCl_2$, 2; NH_4NO_3, 5; $MgSO_4$, 0.5; $MnSO_4$, 0.01; $FeSO_4$, 0.01; sodium phytate, 2; and agar, 15 (pH 7) at 37 ºC for 2-3 days. Formation of clear zone around the colony indicates phytase production. Phytase positive strains were analysed for phytase activity as described by (Pal Roy et al., 2016). Bacterial strain was grown in phytase production media (PPM) (g L^{-1}): glucose, 5.0; yeast extract, 3.0; $MgSO_4$, 0.5; KCl, 0.5 g; $CaCl_2$,0.1; sodium phytate,1.0 (pH 6) for 72 h at 37 ºC. Culture aliquot was withdrawn at 24 h intervals for 72 h and centrifuged at 10,000 rpm for 10 min and the supernatant was used as crude enzyme extract (CEE). 2 mL of reaction mixture containing 100 mM buffer [citrate buffer for pH 4.5 and 5.5, phosphate buffer for pH 6.5 and 7.5], 1 mM sodium phytate, 200 µl of CEE was incubated at 37 ºC for 30 min followed by addition 10 % (v/v) tri-chloroacetic acid (TCA) to stop the reaction. Then Pi reagent (ammonium molybdate, 0.5 %; concentrated H_2SO_4, 0.5 N; and ascorbic acid 2 %) was added to the reaction mixture and kept for 10 min at 37 ºC followed by the monitoring absorbance at 610 nm. The amount of Pi released from phytate was

determined by using Pi standard curve (Fig. 2.3). One unit (U) of phytase activity represents 1 µmol of Pi released per min under standard assay conditions.

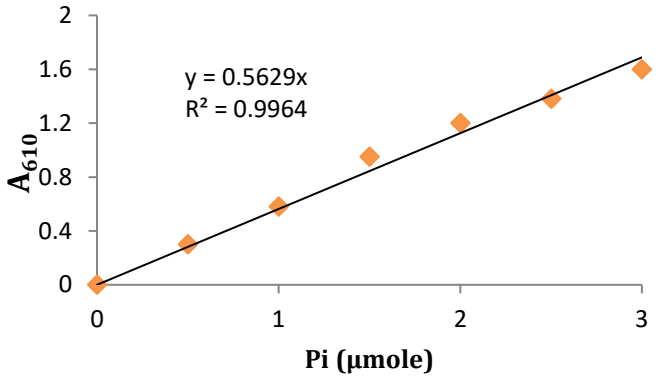


Fig.2.3. Standard curve of inorganic phosphate (Pi)

2.2.4.5. Solubilisation of Zinc

The ability of the bacterial cultures to solubilise zinc was done by the method of Dinesh et al. (2018). Bacterial isolate grown on NB for 24 h was spot inoculated on zinc solubilizing medium (ZSM) containing (g L^{-1}) glucose 10, $Zn_3(PO_4)_2$ [Zinc phosphate (ZP)] or $ZnCO_3$ [Zinc carbonate (ZC)],10; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.2; KCl, 0.2; Agar 15, pH 7 and incubated at 37 ºC for 5 days. Zinc solubilisation index (ZSI) was calculated applying the following equation:

$$ZSI = \frac{\text{Diameter of Zinc solubilisation zone (mm)}}{\text{Growth diameter of spot inoculants (mm)}} \quad \text{(Eq 2)}$$

2.2.4.6. Production of Indole Acetic Acid (IAA)

The production of IAA was determined by following the method of Patten and Glick (2002). Bacterial culture was grown in 100 mL of Luria-Bertani (LB) broth with (0.2 mg mL^{-1}) or without tryptophan at 37 °C under shaking at 120 rpm for 2 days. Then the culture was centrifuged at 10,000 rpm for 10 min and 1 mL of CFE was mixed with 2 mL of Salkowski's reagent (2 % 0.5 $FeCl_3$ in 35 % Perchloric acid solution) and incubated for 30 min at RT (25 ºC). Development of pink colour indicated IAA production and its optical density was recorded at 530 nm. Concentration of IAA was estimated using standard curve prepared with 1-20 µg mL^{-1} IAA (Fig 2.4).

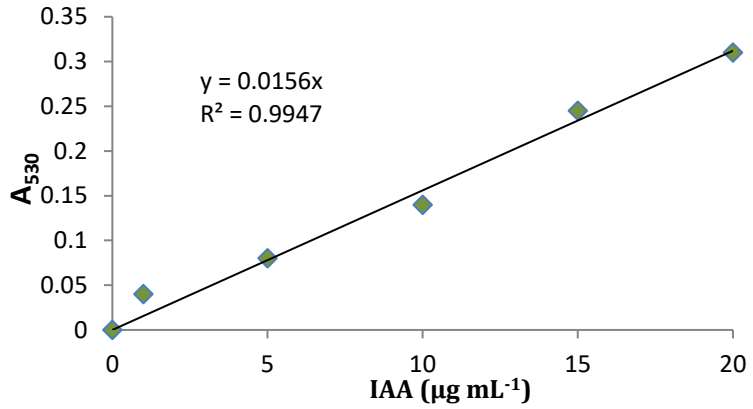


Fig 2.4. Standard curve of IAA

2.2.4.7. Screening for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase

Qualitative screening for ACC deaminase production by the isolates was performed by the method of Kumar et al. (2012). Bacterial isolate was grown on LB medium at 37 ^{o}C and mid log phase cells were harvested by centrifugation at 6000 rpm for 10 min. The bacterial pellet was washed twice with sterile saline and resuspended in 0.5 mL of 0.85% saline. The resuspended cells were spot inoculated on minimal media plates containing ACC as sole N-source and incubated at 37 ^{o}C for 3 days. Medium with ammonium sulphate as N-source served as control.

2.2.5. Identification of the bacterial isolates

2.2.5.1. Morphological and biochemical characterization

For morphological characterization of the bacterial isolates gram staining, growth pattern, motility test, spore formation test were done. Growth pattern of the bacteria was monitored in nutrient agar media for identification of their aerobic/anaerobic nature; motility test was performed in the sulphide indole motility agar medium; spore formation capability was checked by endospore staining using malachite green staining; and gram staining was performed to classify the microorganisms in specific genera. The biochemical characterisation studies included catalase test, Voges-Proskauer test, methyl red test, urease test, nitrate reduction test, oxidase test, citrate utilization, indole test, starch hydrolysis test, casein hydrolysis, gelatine liquefaction test, lipid hydrolysis, growth at 45 ^{o}C, growth in 7 % NaCl, fermentation of sugars (glucose, fructose, mannitol, lactose, sucrose, maltose, starch, xylose, sorbitol, mannose) (Smibert and Kreig, 1994).

2.2.5.2. Molecular identification of the isolates

2.2.5.2.1. Isolation of genomic DNA from bacterial isolates

Genomic DNA was isolated by Murmur's method (Murmur 1961). Bacterial culture was inoculated in LB broth and incubated for overnight at 37 ^{o}C and then centrifuged at 8,000 rpm for 10 min at 4 ^{o}C. The cell pellet was washed with 0.1M EDTA: 0.15 M NaCl

solution (1:1) followed by centrifugation at 10,000 rpm for 5 min. Cell pellet was resuspended in 2-3 mL of 0.1 M EDTA: 0.15 M NaCl solution and was stored at -20 °C for 4 h. Frozen cells were thawed at 55 ^{o}C in water bath till dissolution and incubated with 50 µg mL^{-1} solution of lysozyme (prepared in 0.1 M Tris-HCl pH 8) at 37 °C for 30 min. To the cell lysate, SDS was added and incubated at 55 °C for 15 min. The resulting mixture was then treated with proteinase K (4 µg mL^{-1}) at 55°C for 30 min. Genomic DNA was purified from the lysate by sequential extraction with equal volume of Tris-saturated phenol (pH 8), Tris-saturated phenol:chloroform (1:1) and chloroform (Sambrook et al., 1989). DNA was precipitated from the aqueous phase by adding double volume of absolute ethanol followed by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA pellet was washed with 75 % ethanol, air dried and dissolved in TE buffer [10 mM Tris HCl and 1 mM EDTA (pH 8.0)].

2.2.5.2.2. Agarose gel electrophoresis of DNA

1% agarose in 1X Tris-acetate EDTA (TAE) buffer (1 litre of 50X solution contains 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA) was melted, cooled to 50-60 °C and then supplemented with 5µg mL^{-1} ethidium bromide (EtBr). The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. DNA sample was mixed with 1X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in distilled water) and loaded onto the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer at 50 V. DNA band was visualized on a UV-Transilluminator.

2.2.5.2.3. PCR amplification of 16S rRNA

PCR amplification of 16S rRNA gene was done by using bacterial genomic DNA as template. The reaction mixture in total volume of 25 µl containing 12.5 µl ultrapure water, 5µl 5X Go Tag Green buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2µl $MgCl_2$ (2mM), 1µl dNTPs (10 mM), 1 µl 27F forward primer (10 µM, 5'-AGAGTTTGATCCTG GCTCAG-3'), 1 µl 1492R reverse primer (10 µM, 5'-TACGGTTACCTTGTTACGACT T-3'), 2 µl genomic DNA (50 ng) and 0.50 µl Taq polymerase enzyme (5 U μl^{-1}, Promega, USA). PCR was performed with initial denaturation at 94 ^{o}C for 5 min followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and extension at 72 ^{o}C for 1 min and then a final extension at 72 ^{o}C for 7 min. The PCR product was resolved on 1% agarose-TAE gel along with 500 bp DNA ladder. The bright band of around 1500 bp was cut from the gel, and then extracted and purified using gel extraction kit (QIAGEN, India). The purified PCR product was cloned in pGEM-T easy vector (Promega, USA) using the manufacturer's instruction. The ligation mix consisted of 5 µl of 2X ligation buffer, 1 µl pGEM-T vector, 2 µl PCR product and T4 DNA ligase, which after overnight incubation at 10^{o}C was transformed into competent *Escherichia coli* JM109. The transformants were selected in LB-medium containing ampicillin (50 µg mL^{-1}), X-gal (80 µg mL^{-1}) and IPTG (0.5 mM). The recombinant plasmid was isolated from the transformed cell by the Alkaline lysis method (Birnboim and Doly, 1979). The

nucleotide sequence of the 16S rRNA gene was determined by dideoxy chain termination methods using vector based T7 and SP6 primers.

2.2.5.2.4. Phylogenetic analysis

The phylogenetic relationship of the bacterial isolates were determined by comparing their 16S rRNA sequences, with closely related neighbour sequences retrieved from the GenBank database of the National Centre for Biotechnology Information (NCBI), via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Phylogenetic analysis was performed by using the software package MEGA 4 (Tamura et al., 2007) after obtaining multiple alignments of the data available from public database by Clustal W (Thompson et al., 1994). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 replicates (Russo and Selvatti, 2018).

2.2.6. Statistical analysis

All the experiments were performed in triplicate. The data represented as mean along with standard error and their significance was further checked by 1 and 2 way analysis of variance (ANOVA) using SPSS version 16 (Saha and Ghosh, 2014). Homogeneity of mean was further analysed by Duncan's multiple range test (DMRT) at a level of significance $p< 0.05$ (Glen, 2017). Selection of the potent PGPR strains were done using Principal component analysis (Jolliffe and Cadima, 2016) of the qualitative plant growth promoting (PGP) traits of those isolates which showed at least 4 PGP traits, using SPSS version 16.

2.3. Result

2.3.1. Isolation of rhizosphere bacteria and qualitative screening of PGP traits

Analysis of the rhizosphere soil sample showed approximately10^8 cfu g^{-1} of soil on nutrient agar plate. Initially 82 bacterial isolates were screened based on morphologically distinct characteristics, of which 27 isolates showing the ability to fix atmospheric N were further tested for other PGP traits, like IAA production, inorganic phosphate solubilisation, organic phosphate solubilisation, Zn solubilisation, ACC deaminase production. Among the N fixing isolates, 12 produced IAA (44 %), 22 solubilised DCP (81 %), 6 solubilised TCP (22 %), 16 produced phytase (59 %), 14 and 16 isolates showed zone of solubilisation in media with ZP (52 %) and ZC (59 %), respectively, 10 isolates showed positive for ACC deaminase (37 %). The distribution of PGP traits between the bacterial isolates has been shown Table 2.1. The strain RS3, showing the positive result for almost all PGP traits (Table 2.1).

The PGP traits shared by the bacterial isolates are represented by venn-diagrams (Fig 2.5a & b, Fig 2.6a & b, Fig 2.7a & b). From the results, it is apparent that three isolates shared N_2 fixation, IAA production and ACC deaminase production (Fig 2.5a) traits; seven isolates were common in sharing the traits of N_2 fixation and Pi (DCP) solubilisation; two

were common in sharing the traits of N_2 fixation, Pi (DCP) and Pi (TCP) solubilisation; two isolates were common in sharing the traits of N_2 fixation, DCP, TCP and organic

Table 2.1.
Screening of PGPR for plant growth promotion attributes of Nitrogen fixation, Phosphate solubilisation, Zinc solubilisation, IAA production, ACC deaminase. ('+' = positive for PGP traits and '–' = negative for PGP traits)

PGPR	N_2 fixation	Phosphate solubilization			Zinc solubilization		IAA production	ACC deaminase
		Inorganic		Organic				
		DCP	TCP		$ZnCO_3$	$ZnPO_4$		
RS2	+	-	-	+	+	+	-	+
RS3	+	+	+	+	+	+	+	+
RS5	+	+	-	+	-	+	-	-
RS6	+	+	-	+	+	-	-	+
RS7	+	+	+	-	-	-	+	-
RS9	+	+	-	-	+	+	+	-
RS10	+	+	-	-	-	+	+	+
RS11	+	+	-	+	-	-	+	-
RS14	+	-	-	+	+	+	-	-
RS16	+	+	-	-	-	+	-	+
RS20	+	+	+	+	-	-	-	-
RS23	+	+	-	+	+	-	+	-
RS26	+	+	-	+	+	+	+	+
RS31	+	+	-	-	-	+	+	-
RS39	+	+	-	+	+	-	-	-
RS46	+	+	-	-	+	-	+	-
RS48	+	-	+	-	+	-	+	-
RS49	+	+	-	+	+	-	+	-
RS51	+	+	-	-	-	+	+	-
RS53	+	+		+	+	-	-	+
RS59	+	+	+	-	-	+	-	+
RS60	+	+	-	+	-	-	-	+
RS61	+	-	-	+	+	+	-	-
RS65	+	-	+	-	+	-	-	+
RS71	+	+	-	+	-	+	-	-
RS74	+	+	-	-	+	+	-	-
RS77	+	+	-	+	+	-	-	-

phosphate (OP) solubilisation; eleven bacteria shared N_2 fixation, DCP and OP solubilisation properties; two isolates shared the traits of N_2 fixation and Pi (TCP) solubilisation; three isolates were common in sharing N_2 fixation and OP solubilisation attributes (Fig 2.5b); seven isolates shared the traits of N_2 fixation, zinc carbonate (ZC) and zinc phosphate (ZP) solubilisation (Fig 2.6a); one isolate showed ZC, ZP, DCP, TCP and OP solubilisation properties (Fig 2.6b); one isolate showed the traits of IAA, OP, TCP and DCP solubilisation (Fig 2.7a); four isolates were common in sharing ZC solubilisation and IAA production; one isolate showed ZC and ZP solubilisation, IAA production and ACC deaminase production; and two isolates shared ZC solubilisation,

IAA production and ZP solubilisation traits (Fig 2.7b). Isolates showing the positive result in the qualitative screening were further evaluated quantitatively for their PGP trait.

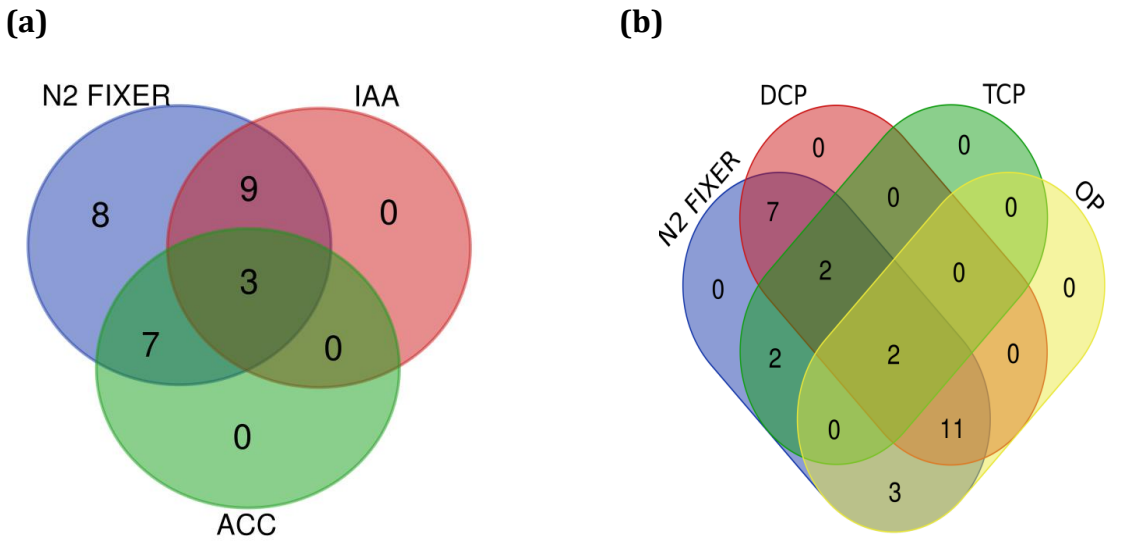


Fig 2.5. Distribution of PGP traits among PGPR isolates as shown by venn-diagram (a) N_2-fixation, IAA production and ACC deaminase production, (b) N_2-fixation, Dicalcium phosphate (DCP), Tricalcium phosphate (TCP) and Organic phosphate (OP) solubilisation.

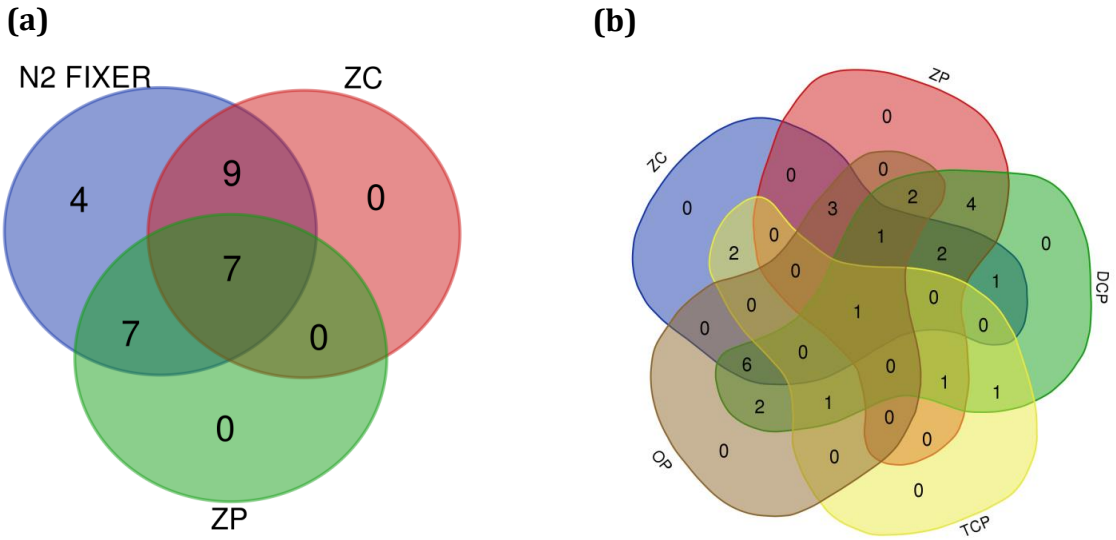


Fig 2.6. Distribution of PGP traits among PGPR isolates as shown by venn-diagram (a) N_2-fixation, Zinc carbonate (ZC) and Zinc phosphate (ZP) solubilisation (b) Zinc carbonate (ZC) and Zinc phosphate (ZP), Di-calcium phosphate (DCP), Tri-calcium phosphate (TCP) and Organic phosphate (OP) solubilisation.

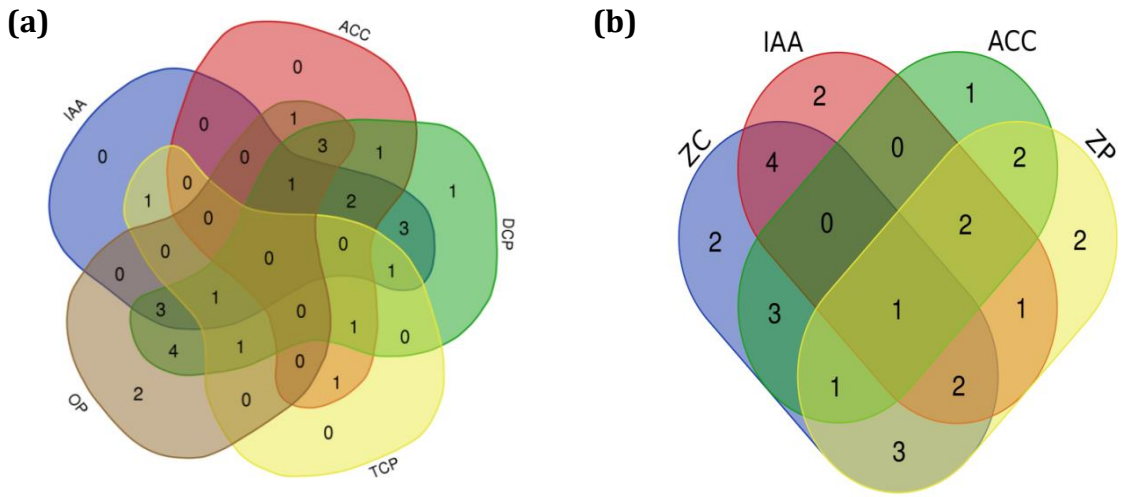


Fig 2.7. Distribution of PGP traits among PGPR isolates as shown by venn-diagram (a) Indole acetic acid (IAA) production, ACC deaminase production, Di calcium phosphate (DCP), Tri calcium phosphate (TCP) and Organic phosphate (OP) solubilisation (b)Indole acetic acid (IAA) production, ACC deaminase production, Zinc carbonate (ZC) and Zinc phosphate (ZP) solubilisation among the isolates.

2.3.2. Morphological and biochemical identification of the isolated PGPR

Twenty seven isolated potent plant growth promoting rhizobacteria bacteria (PGPR) were identified based on their colony morphology in nutrient agar plates, gram nature and biochemical reaction up to genus level. The biochemical test results of all the 27 PGPR isolates are depicted in the Table 2.2 and from the result it can be found that **one** of the isolates belongs to the genus *Cedecea* (RS3), **two** bacteria affiliated to the genus *Streptococcus* (RS9, RS51), **seven** to the genus *Bacillus* (RS2, RS5, RS16, RS23, RS39, RS53, RS60), **five** to the genus *Pseudomonas* (RS6, RS7, RS1, RS46, RS49), **two** of the isolates are *Klebseilla* sp. (RS26, RS71), **four** of the isolates are *Acinetobacter* sp. (RS31, RS59, RS65 & RS77), **three** of the isolates belongs to the genus *Azotobacter* (RS10, RS14 & RS19) and **three** of the isolates belongs to the genus *Burkholderia* (RS48, RS61 and RS74).

2.3.3. Quantitative screening of PGP traits

2.3.3.1. N_2-fixation and ammonia production

The first criterion in screening of PGPR in this study was to isolate bacterial strains with the ability to fix atmospheric N_2. Among the 82 rhizospheric isolates 27 selected isolates were able to grow in N-free Asbhy's agar media indicating their ability to fix N_2 to ammonia. Among the 27 isolates three strains RS3, RS26 and RS23 were found to form pellicle in the JNFb semi-solid N-free medium which further confirmed them as free living N_2-fixers. All these 27 selected isolates were further screened for quantitative

Table 2.2
Morphological and biochemical characteristics of the Plant growth promoting rhizobacterial isolates

Sl. No	Isolates	Morphological and Biochemical characteristics	Identity of the bacteria
1	RS2	Gram positive, rod shaped, colony diameter 3.2 mm, white, convex and irregular margin colony, spore formation(+ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (G), glycerol (G), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG)	*Bacillus* sp.
2	RS 3	Gram negative, rod shaped, colony diameter 1.2 mm, yellow, small and smooth, slimy colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (AG), glycerol (AG), maltose (+ve), glucose (AG), xylose (AG), arabinose (AG), cellobiose (AG), sucrose (G), fructose (G)	*Cedecea* sp.
3	RS5	Gram positive, rod shaped, colony diameter 3.3 mm, creamy white, convex colony, spore formation (+ve), motility (+ve), growth under aerobic condition (+ve), Starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalse (+ve), oxidase (-ve), lipase (+ve), citrate utilization (-ve), Voges- Proskauer test (-ve), methyl red (+ve), indole (-ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (G), glucose (AG), xylose (AG), arabinose (A), cellobiose (AG), fructose (A), sorbitol (A)	*Bacillus* sp.
4	RS6	Gram negative, rod shaped, colony diameter 1.2 mm, creamy yellow, small and rough colony, spore formation (-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (-ve), glycerol (AG), maltose (-ve), glucose (G), xylose (A), arabinose (A), cellobiose (AG)	*Pseudomonas* sp.

5	RS 7	Gram negative, rod shaped, colony diameter 1.4 mm, yellow, small and smooth colony, spore formation (-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (+ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), indole (-ve), nitrate reduction (+ve), acid and gas production from starch (G), lactose (-ve), glycerol (AG), maltose (+ve), glucose (G), xylose (A), arabinose (A), cellobiose (AG), sorbitol (G)	*Pseudomonas* sp.
6	RS 9	Gram negative, rod shaped, colony diameter 3 mm, watery to translucent, mucoid, conical and entire margin colony, growth period 3-5 days, spore formation (-ve), motility (-ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from glucose (AG), arabinose (AG), tryptophan (+ve), mannose (AG), mannitol (AG)	*Rhizobium* sp.
7	RS 10	Gram negative, rod shaped, colony diameter 1.1 mm, white, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalse (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (+ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (AG), cellobiose (AG), sucrose (G), fructose (AG)	*Azotobacter* sp.
8	RS 11	Gram negative, rod shaped, colony diameter 1mm, yellow, small and smooth colony, spore formation(-ve), motility (-ve), growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (+ve), catalse (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), indole (-ve), nitrate reductase (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (+ve), glucose (G), xylose (AG), arabinose (AG), cellobiose (AG), sorbitol (G), Fructose (G)	*Pseudomonas* sp.

9	RS 14	Gram negative, rod shaped, colony diameter 1.2 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (+ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (+ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (AG), cellobiose (AG), sucrose (G), fructose (AG)	*Azotobacter* sp.
10	RS16	Gram positive, rod shaped, colony diameter 2.8 mm, creamy white, convex and irregular margin colony, spore formation (+ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalse (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (AG)	*Bacillus* sp.
11	RS20	Gram negative, rod shaped, colony diameter 2.8 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (+ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), Methyl red (+ve), Indole (+ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (G), cellobiose (AG),sucrose (G), fructose (AG)	*Azotobacter* sp
12	RS23	Gram positive, rod shaped, colony diameter 2.8 mm, creamy white, convex and irregular margin colony, spore formation (+ve), motility (+ve), Growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), Indole (-ve) nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (AG)	*Bacillus* sp.

13	RS26	Gram negative, rod shaped, colony diameter 1.2 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (+ve), methyl red (-ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (AG), cellobiose (AG), sucrose (G), Fructose (AG)	*Klebseilla* sp.
14	RS31	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (-ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (NF), lactose (NF), glycerol (NF), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G), sucrose (NF), Fructose (NF)	*Acinetobacter* sp.
15	RS39	Gram positive, rod shaped, colony diameter 3.4 mm, creamy white, convex and entire margin colony, spore formation(-ve), motility (+ve), Growth under aerobic condition (+ve), starch hydrolysis(+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (G), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (G)	*Bacillus* sp.
16	RS46	Gram negative, rod shaped, colony diameter 1.2 mm, creamy yellow, small and rough colony, spore formation (-ve), motility (+ve), Growth under aerobic condition (+ve), starch hydrolysis(-ve),casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), indole (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose(-ve), glycerol (AG), maltose (-ve), glucose (G), xylose (A), arabinose (A),cellobiose (AG), sorbitol (G)	*Pseudomonas* sp.

17	RS48	Gram negative, cocco bacilli shaped, colony diameter 1.1 mm, red, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (NF), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (NF), cellobiose (AG), sucrose (G), fructose (AG)	*Burkholderia* sp.
18	RS49	Gram negative, rod shaped, colony diameter 1.2 mm, creamy yellow, small and rough colony, spore formation(-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (-ve),casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges- Proskauer test (-ve), methyl red (+ve), indole (-ve), nitrate reductase (-ve), acid and gas production from starch (G), lactose(-ve), glycerol (AG), maltose (-ve), glucose (G), xylose (A), arabinose (A), cellobiose (AG),	*Pseudomonas* sp.
19	RS51	Gram negative, rod shaped, colony diameter 3.2 mm, watery to translucent, mucoid, flat and entire margin colony, growth period 3-5 days, spore formation (-ve), motility (-ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from glucose (AG), arabinose (AG), tryptophan (-ve), mannose (AG), mannitol (AG)	*Rhizobium* sp.
20	RS53	Gram positive, rod shaped, colony diameter 2.8 mm, creamy white, convex and entire margin colony, spore formation (-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (+ve), methyl red (-ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (AG)	*Bacillus* sp.

21	RS59	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), Motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (-ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (NF), lactose (NF), glycerol (AG), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G) ,sucrose (NF), fructose (NF)	*Acinetobacter* sp.
22	RS60	Gram positive, rod shaped, colony diameter 3.3 mm, creamy white, convex colony, spore formation (+ve), motility (-ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (G), glucose (AG), xylose (AG), arabinose (A), cellobiose (AG), fructose (A), sorbitol (AG)	*Bacillus* sp.
23	RS61	Gram negative, cocco bacilli shaped, colony diameter 1.1 mm, red, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (NF), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (NF), cellobiose (AG), sucrose (G), fructose (AG)	*Burkholderia* sp.
24	RS65	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalse (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (NF), lactose (NF), glycerol (AG), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G), sucrose (NF), fructose (NF)	*Acinetobacter* sp.

25	RS71	Gram negative, rod shaped, colony diameter 1.1 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, Growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (+ve), methyl red (-ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (G), glucose (AG), xylose (AG), arabinose (AG), cellobiose (G), sucrose (G), fructose (G)	*Klebseilla* sp.
26	RS74	Gram negative, cocco bacilli shaped, colony diameter 1.1 mm, red, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (+ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (NF), glycerol (AG), maltose (AG), glucose (AG), xylose (AG), arabinose (NF), cellobiose (AG), sucrose (G), fructose (NF)	*Burkholderia* sp.
27	RS77	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (NF), lactose (NF), glycerol (AG), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G), sucrose (NF), fructose (NF)	*Acinetobacter* sp.

A= Only acid produced, AG= Acid and gas produced, G= Only gas produced, NF= Not found,
+ve = present , -ve = absent

production of ammonia in N-free Ashby's broth. The result in Fig 2.8 shows that the isolates RS3, RS23, RS26 and RS51 produced more than 6 μg mL^{-1} of ammonia with significantly higher production by RS3 (9.52 μg mL^{-1}) and RS26 (10.13 μg mL^{-1}).

2.3.3.2. Phosphates solubilisation

Among the nitrogen fixers, twenty-two were found to solubilise DCP, six solubilised TCP, and sixteen produced phytase. The phosphate solubilisation index (PSI) of DCP solubilising bacteria are shown in Fig 2.9a & b. It can be seen that isolates RS3, RS7, RS10, RS26, RS46 and RS49 displayed PSI $\geq$ 2. The PSI of RS3, RS23 and RS26 positively correlated with their ability to solubilise DCP.

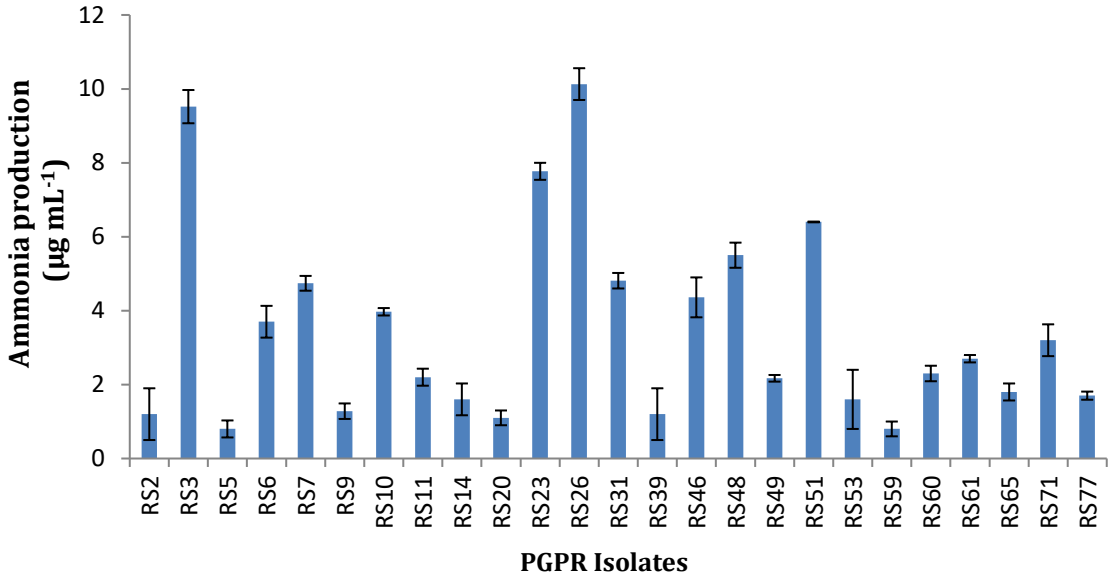


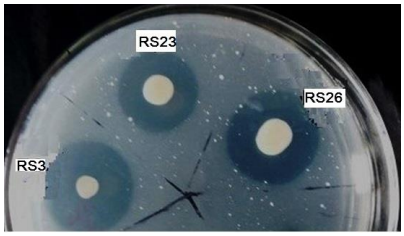
Fig 2.8. Production of ammonia by rhizosphere isolates in N-free Asbhy's broth media. Data were represented as triplicate of mean±standard deviation.

The liberation of Pi by RS3, RS23, RS26 and RS49 were recorded to be 49, 40, 26 and 30 µg mL^{-1}, respectively (Fig 2.9c). The release of Pi was found to be associated with decline in the medium pH from neutral to 3, 4, 5.5 and 5 in case of RS3, RS23, RS26 and RS49, respectively. The organic phosphate (phytate) solubilisation attribute was determined by inoculating and incubating the isolates on PSM plates containing sodium phytate. Sixteen bacteria were found to utilize phytate phosphorous. Further their ability to produce the enzyme phytase was also checked and it was found that almost all the sixteen bacteria produced phytase with significantly higher activity of RS3, RS23, RS26 and RS49 in the pH range 5.5 to 6.5 (Fig 2.10).

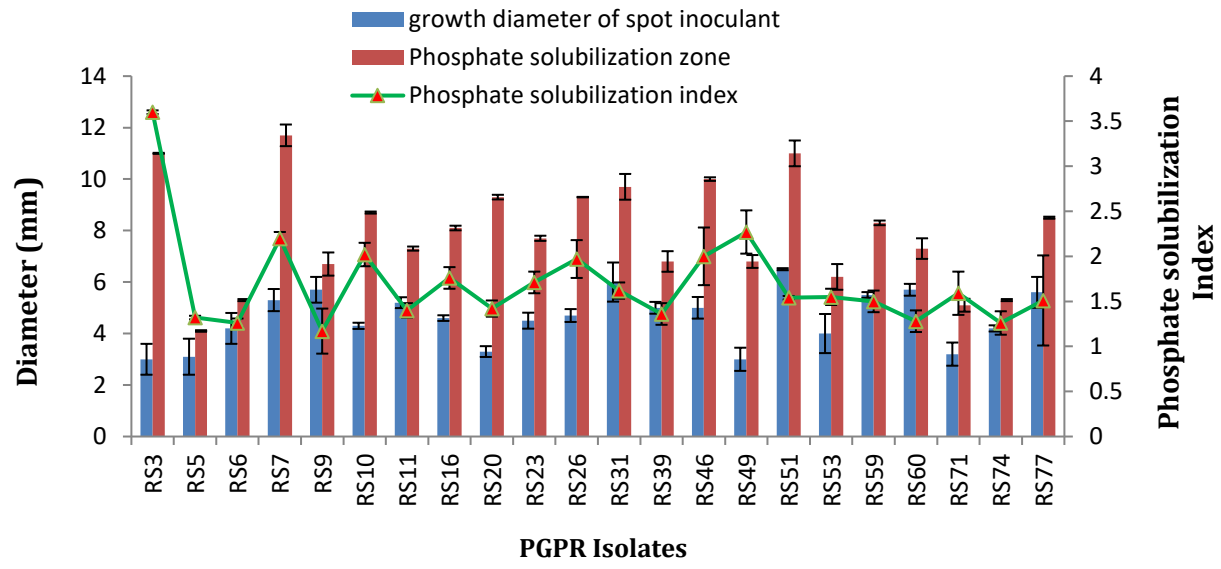
2.3.3.3. Zinc solubilisation

The property of zinc solubilisation of the selected isolates was determined by cultivating them on agar media containing either $ZnCO_3$ or $Zn_3(PO_4)_2$. In total sixteen isolates were found to solubilise $ZnCO_3$, whereas fourteen isolates were recorded to solubilise $Zn_3(PO_4)_2$ (Fig 2.11a). PGPR isolates RS2, RS3, RS6, RS9, RS14, RS23, RS26, RS39, RS46, RS48, RS49, RS53, RS60, RS61, RS71, RS77 were found to solubilise insoluble $ZnCO_3$ whereas, RS2, RS3,RS5, RS9, RS10, RS14, RS16, RS26, RS31, RS51, RS59, RS61, RS71 and RS74 solubilised $Zn_3(PO_4)_2$. The result in the Fig 2.11b shows the zinc solubilisation index (ZSI) of the $ZnCO_3$ solubilising isolates. The isolates RS3, RS9 and RS26 showed ZSI of 2.34, 2.06 and 2.07, respectively.

(a)



(b)



(c)

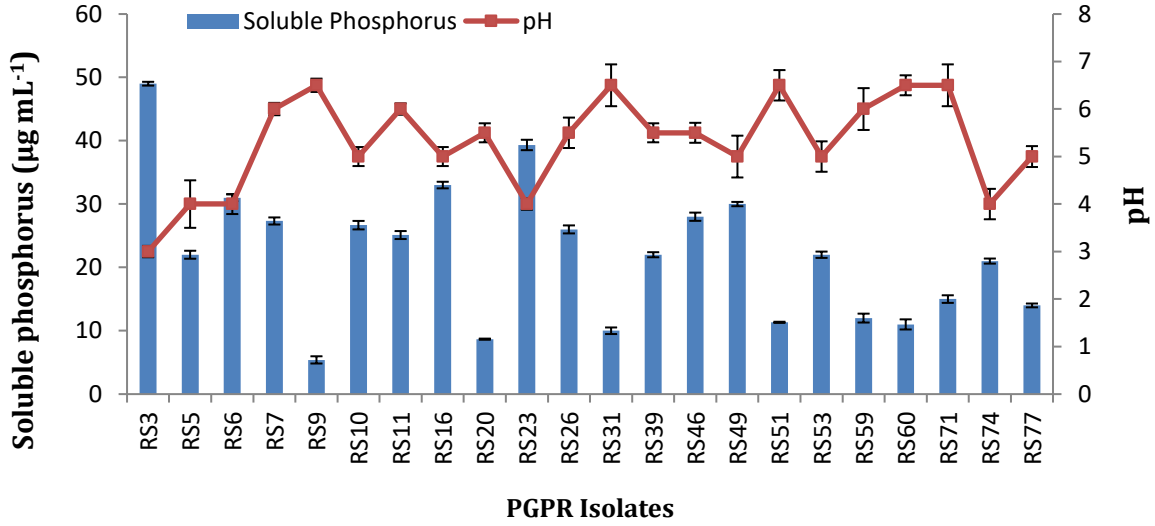


Fig 2.9. (a) Phosphate solubilisation zone of di calcium phosphate (DCP) formed by the PGPR isolates RS3, RS23 and RS26 (b) Phosphate solubilization index (PSI) formed by PGPR isolates on Pikoskaya's agar medium, Diameter (mm) on Y axis refers to the colony growth and diameter of phosphate solubilisation zone and (c) Quantitative estimation of inorganic phosphate liberated during solubilisation of dicalcium phosphate by selected phosphate solubilizers. Data are triplicates of mean ± SD.

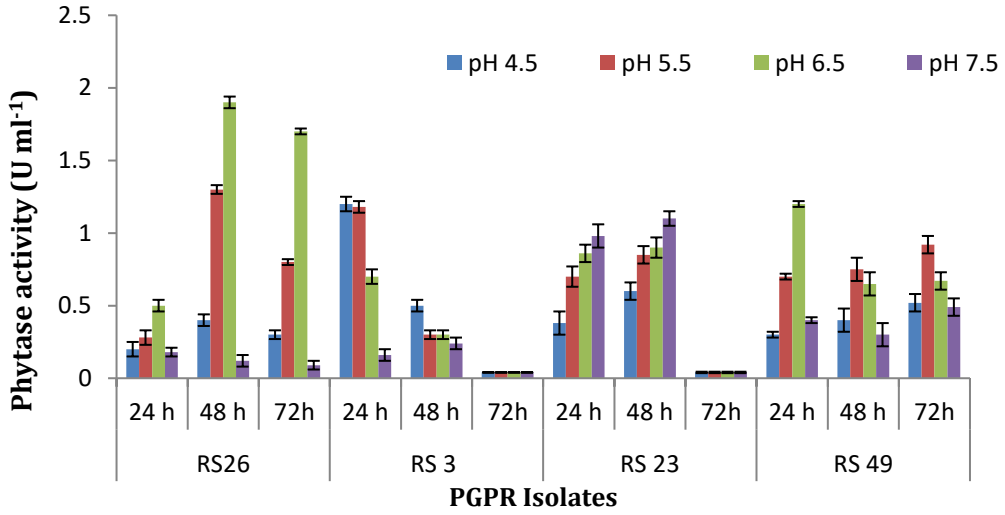


Fig 2.10. Phytase activity of the isolates RS3, RS23, RS26 and RS49 at different pH and different time interval

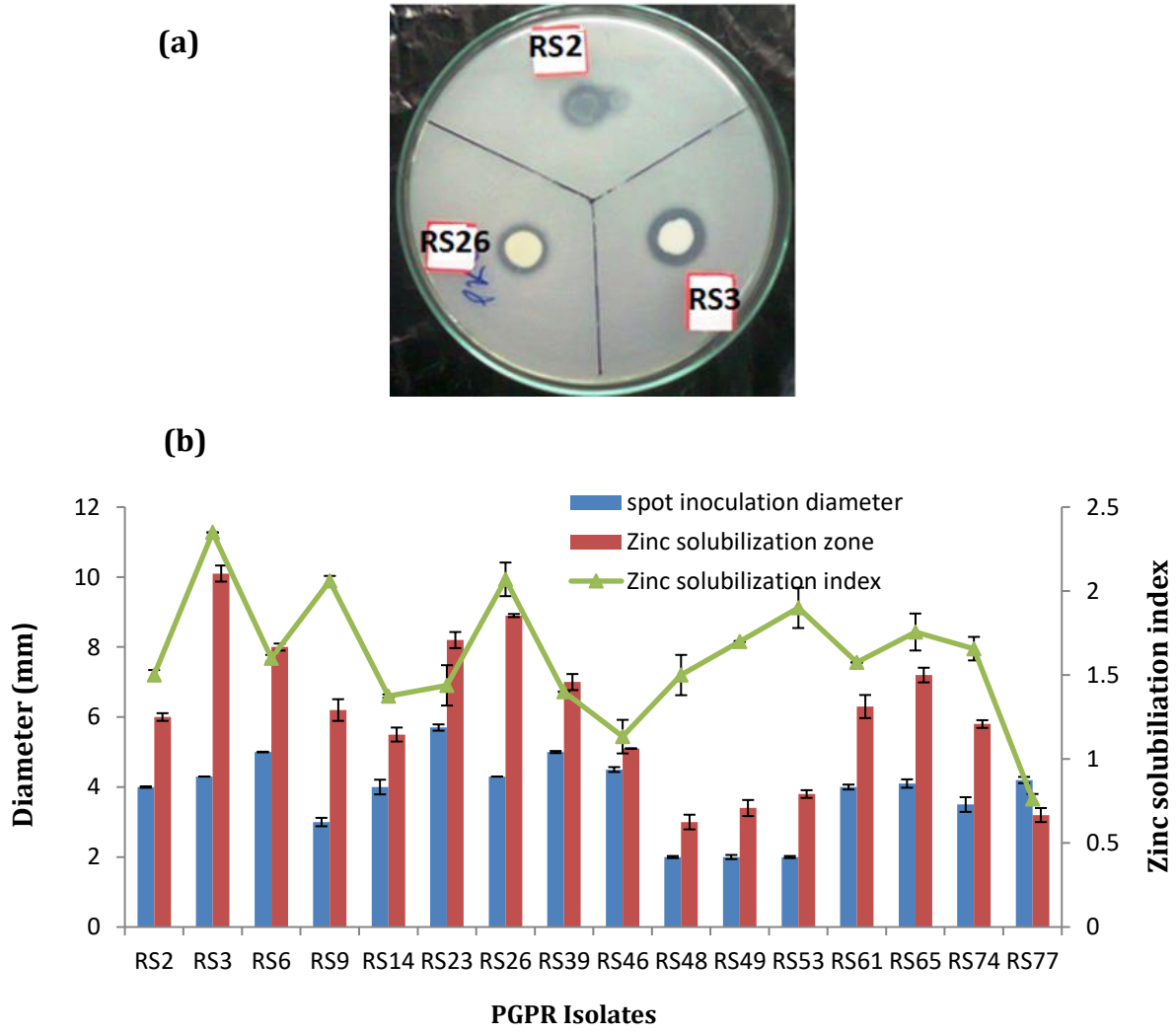


Fig 2.11. Zn solubilisation (a) Solubilisation of insoluble zinc carbonate by RS2, RS3 and RS26 with formation of halo around the colonies (b) Zinc solubilisation index (ZSI) of the PGPR isolates in the zinc carbonate containing media. Diameter (mm) on Y axis refers to the colony growth and diameter of zinc solubilisation zone.

2.3.3.4. Production of IAA

Production of indole acetic acid (IAA) by the PGPR isolates was monitored by growing them in LB broth in presence or absence of tryptophan. The result in Fig 2.12 represents the production of IAA by the isolates. Bacterial strains RS3 and RS26 produced significantly greater quantity of IAA and their respective production levels were 5.06 and 7.13 µg mL^{-1} in absence of tryptophan and 10.13 and 14.51 µg mL^{-1} in presence of tryptophan.

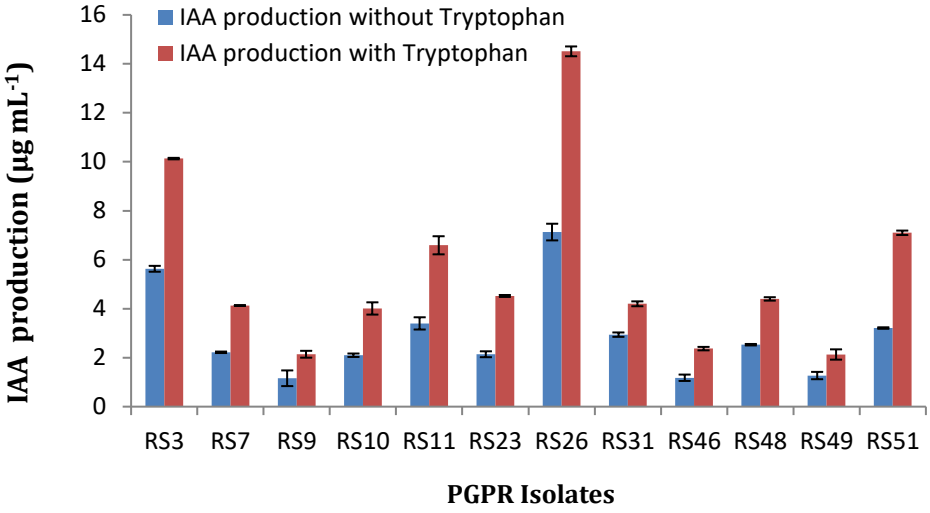


Fig 2.12. Production of Indole acetic acid (IAA) by the PGPR isolates in presence or absence of tryptophan.

2.3.3.5. ACC deaminase synthesis

About 40% of the screened isolate i.e 11 isolates showed positive for ACC deaminase. They can able to grow in minimal media plates containing ACC as sole N-source. RS2, RS3, RS6, RS10, RS16, RS26, RS53, RS59, RS60 and RS65 were ACC deaminase positive.

2.3.3.6. Selection of PGPR strain for application in plant growth

For pot experiment, PGPR isolates (those showing at least 4 PGP traits) were selected based on their presence of maximum PGP attributes as well as by principal component analysis (PCA) using the statistical tool, SPSS (**Fig 2.13**). Combined approach for selection of factors on the basis of Eigen values, scree plot and variance explained criterion of 60%, considered two principal components (PCs), PC1 and PC2 which explain 32.7 and 30.1 % variability, respectively. The major contributory factors to PC1 were ammonia production (0.857), IAA production without tryptophan (0.878), IAA production with tryptophan (0.874), phosphate solubilisation index (0.82), phosphate solubilisation (0.633), zinc solubilisation index (0.642). The microbial strains RS3 and RS26 contributed to PC1 with a correlation coefficient of 0.607 and 0.602, respectively.

The second principal component PC2 represented ACC deaminase production (0.875), phytase (0.76) and organic phosphate solubilisation (0.846). The results of principal component loading plot indicate that microbial strain RS3 and RS26 have significant positive contribution to most of the PGP attributes of PC1 as reflected by the formation of tight cluster by them in the loading plane. PCA thus suggested RS3 and RS26 as potent PGPR and these two strains with more than four PGP attributes were selected for pot trial experiments on mustard (*Brassica campestris* L.) plant.

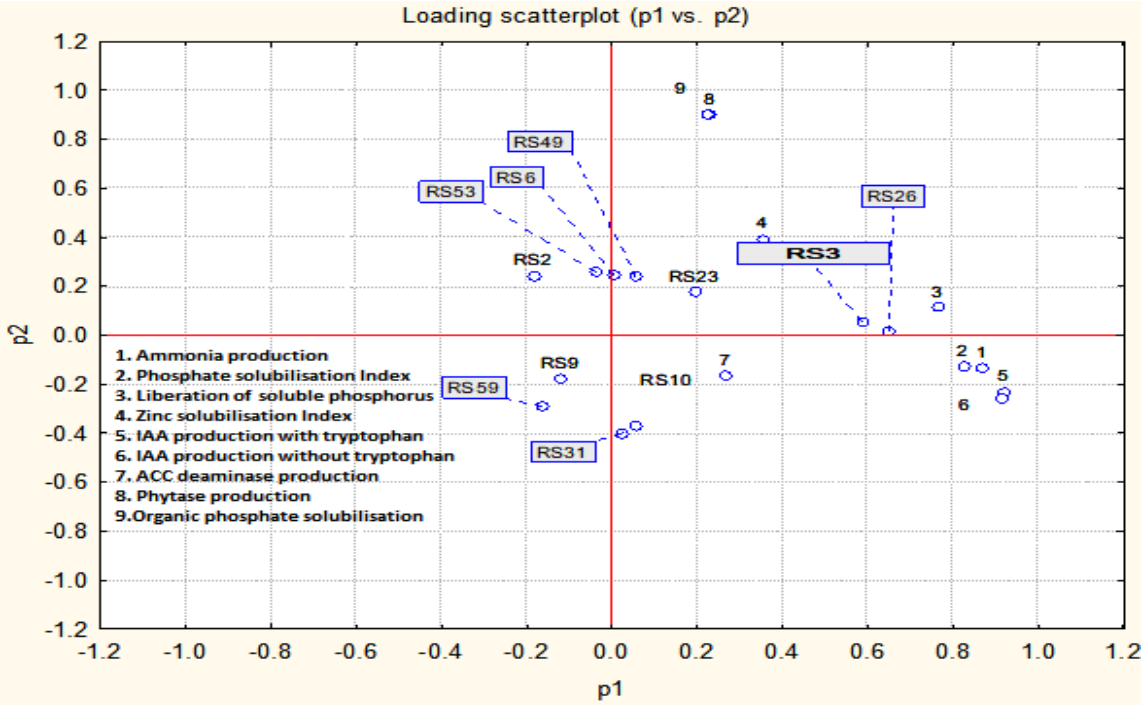


Fig 2.13. Principal component analysis of the plant growth promoting traits (PGP) (presented in Arabic numerical) along with the PGPR isolates, showing the association of isolates with PGP traits.

2.3.3.7. Molecular identification of the isolated potent PGPR

Strain RS3 was further identified using phylogenetic analysis based on 16S rRNA gene sequence comparisons which showed that the isolate RS3 (GenBank accession number **KX101223**) belong to the branch encompassing members of genus *Cedecea* and was most closely related to *Cedecea davisae* with 99% 16S rDNA sequence similarity and hence identified as *Cedecea davisae* RS3 (Fig 2.14).

Another potent strain RS26 was identified by 16S rDNA sequence analysis followed by phylogenetic tree construction (Fig 2.15). The result showed that the strain RS 26 (Gene bank Accession number **MH 819506.1**) is belonging to the genera *Klebseilla* and showed 99% sequence similarity with *Klebsiella pneumoniae* ATCC 13884. Hence, identified as *Klebsiella pneumoniae* RS26.

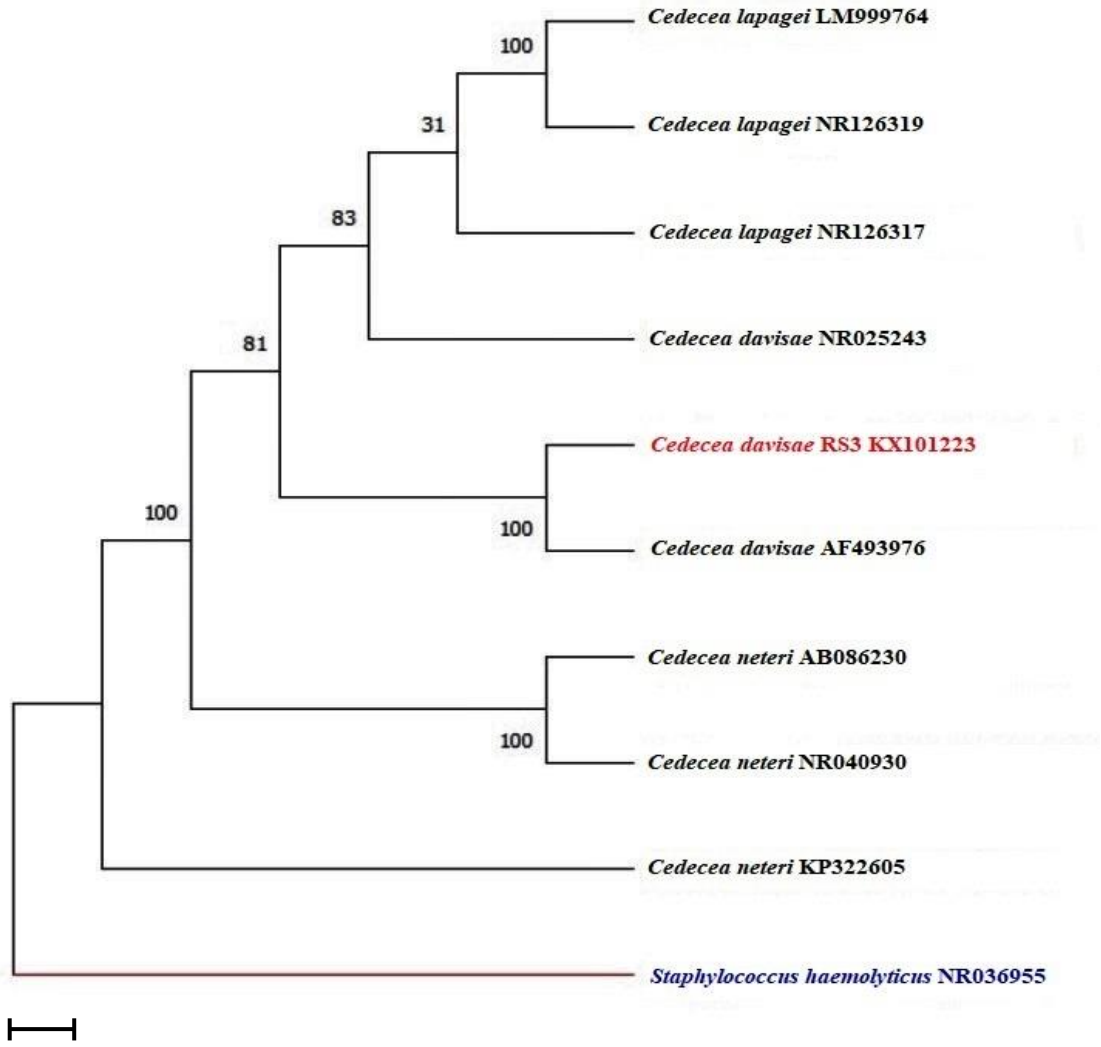


Fig 2.14. Phylogenetic tree created using 16S rRNA gene sequences representing the position of PGPR strain RS3 with the other *Cedecea* species. Bar reflects 1 nucleotide substitution per base. Numbers at nodes shows bootstrap values. At extreme right of the strain names NCBI accession numbers are given in paranthesis.

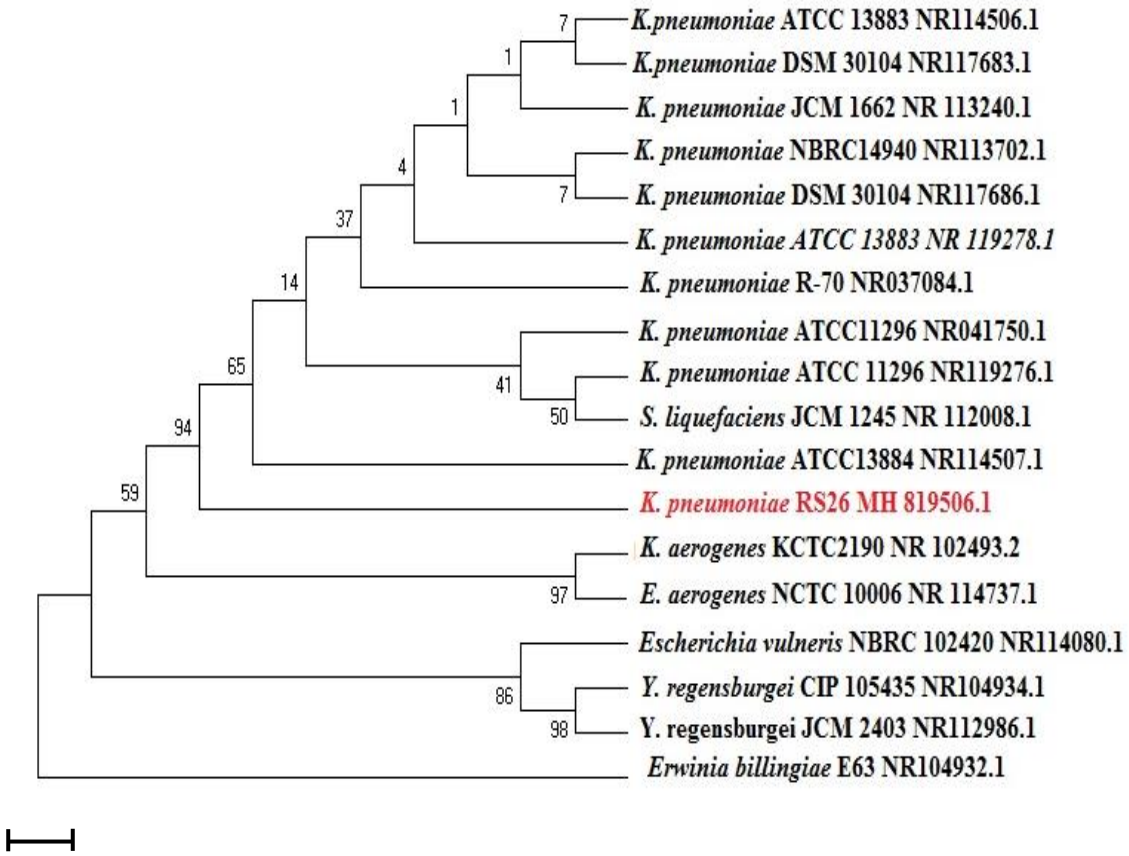


Fig 2.15. Phylogenetic tree created using 16S rRNA gene sequences representing the position of PGPR strain RS26 with the other *Klebsiella* species. Bar reflects 1 nucleotide substitution per base. Numbers at nodes shows bootstrap values. At extreme right of strain names NCBI accession numbers are given.

2.4. Discussion

During past the demand for legumes, cereal crops, and other agriculturally important plants has been met through the increased application of N-fertilizers to the agricultural fields. However, the extensive and indiscriminate use combined with the inefficient uptake of N-fertilizers by the plants is associated with several health and environmental issues. In this context, PGPR having N_2 fixing ability can be a suitable alternative to N-fertilizers or they can be used in combination with lower dose of chemical based N-fertilizer to combat the harmful effects (Dobbelaere et al., 2003; Kennedy et al., 2004). In the present study the rhizobacteria isolated from mustard plant were preliminarily screened for N-fixation attribute. Screening method involving N_2-fixation ability allows further selection of strains with more survival activity in N-deprived soil (Piromyou et al., 2011). Altogether twenty-seven isolates showed positive growth on Asbhy's N-free medium, a differentiating media screening for PGPR with potential biological N_2-fixation ability (Wu et al., 2005; Arruda et al., 2013). They belonged to various genera, like *Bacillus*, *Cedecea*, *Pseudomonas*, *Rhizobium*, *Klebseilla*, *Acinetobacter* and *Burkholderia*. In previous studies several bacterial species belonging to genera like

Paenibacillus, *Bacillus*, *Azospirillum*, *Arthrobacter*, *Achromobacter*, *Burkholderia*, *Chryseobacterium*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Cedecea*, *Pseudomonas*, *Rhizobium*, *Pantoea*, *Herbaspirillum* etc. have been found in the plant rhizosphere and they have been reported to exhibit the potential to promote plant growth and yield (Malik et al., 1997; Mirza et al., 2006; Perin et al., 2006; Beneduzi et al., 2012; Jha and Kumar, 2009, Yadav et al., 2019). Similar to findings of this study, N_2-fixing bacterial strains such as *Bacillus* sp., *Klebsiella* sp., *Azospirillum* sp., *Azotobacter* sp. and *Pantoea* sp. were isolated from rhizospheric soil sample and most of them belongs to class gamma-proteobacteria (Wu et al., 2005; Montanez et al., 2009). In addition, several scientific reports have cited the ability to fix atmospheric N_2 by *Cedecea* sp. (Jeong et al., 2017), *Pseudomonas* sp. (Li et al., 2017; Mirza et al., 2006), *Rhizobium* sp. (Ludwig, 1984), *Acenitobacter* sp. (Kuan et al., 2016), *Burkholderia* sp. (Santos et al., 2002). Further, all N_2-fixer were screened for other PGP traits; and all of them exhibited PGP traits viz. inorganic phosphate solubilisation, organic phosphate solubilisation, zinc solubilisation, IAA production, ACC deaminase production. The distribution of PGP trait among the bacterial genera is presented in the Fig 2.16. Majority of our isolates belong to the class gamma-proteobacteria and few isolates fall under class beta-proteobacteria and firmicutes.

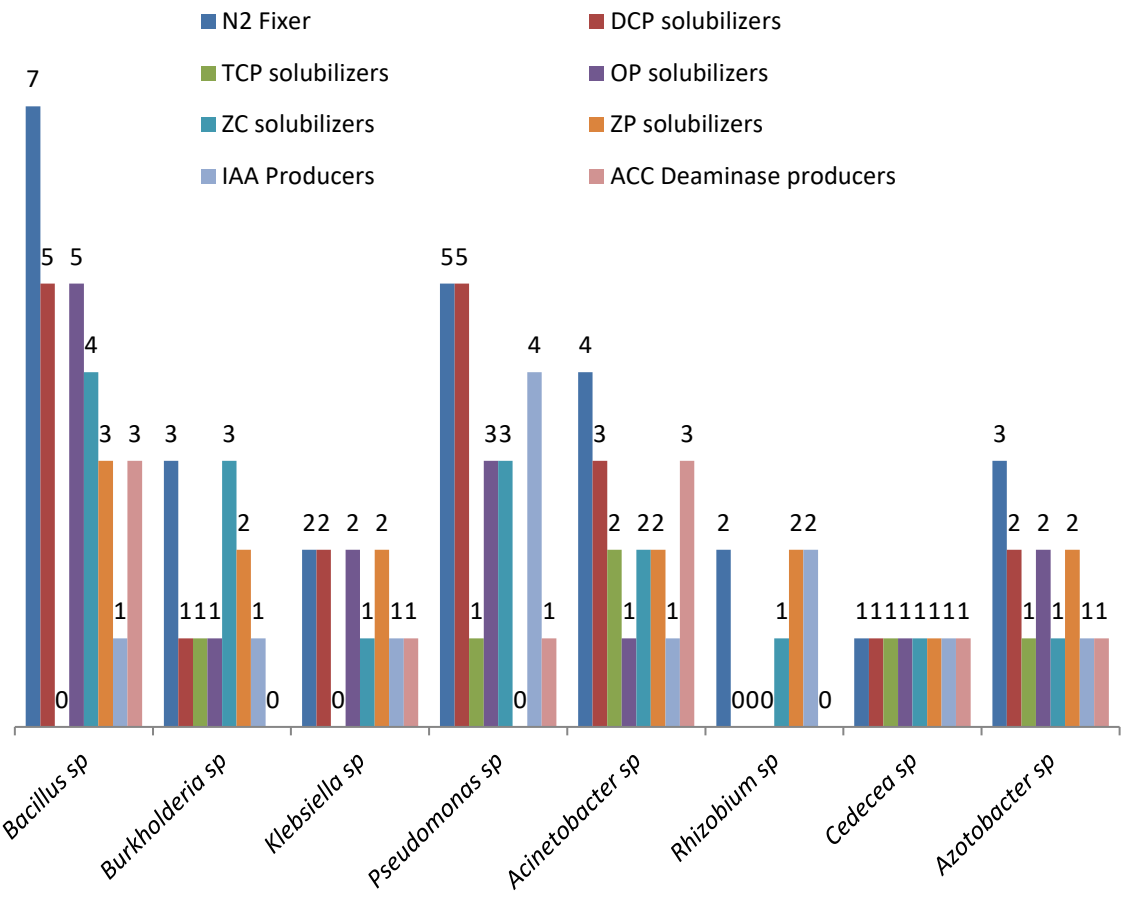


Fig 2.16. Distribution of PGP traits in various bacterial genera. The number of strains obtained for each trait and their respective genera are shown.

Due to its various roles in photosynthesis, respiration, macromolecules biosynthesis and energy transfer, phosphate is a key essential nutrient for plant growth (Huang et al., 2008). In soil the inorganic and organic phosphate compounds are tightly complexed, which are water insoluble and thus poorly available to the plants. Phosphate solubilisation by rhizobacteria is very essential because plant can absorb and assimilate only inorganic soluble phosphate. Release of mineral dissolving compounds, organic acids, protons and extracellular secretion of phosphate hydrolyzing enzymes such as phytases and other phosphatases, are the key to phosphate solubilisation by the phosphate solubilising bacteria (PSB) (Richardson et al., 2011; Pal Roy et al., 2016). In present investigation, bacterial isolates RS3, RS7, RS10, RS23, RS26 and RS49 showed the property of phosphate solubilisation with PSI ≥2 (Fig 2.6a). The Pi release by these bacteria was found to be associated with decrease of media pH from neural to acidic range, which could be due to secretion of organic acids commonly associated with phosphate solubilisation like citric, propionic, gluconic, succinic, oxalic, acetic, formic and lactic acids (Chen et al., 2006; Wei et al., 2018). Furthermore, the phosphate solubilising capability was recorded for most of the isolates belonging to the genera *Bacillus* (Fig 2.9). Similarly, Maheswar and Sathiyavani (2012) reported solubilisation of insoluble phosphates by *B. subtilis* and *B. cereus*. The PGPR strain *Pseudomonas aeroginosa* KUPSB12, showed the PSI index of 2.85 on DCP containing Pikovskaya's agar (Paul and Sinha, 2016) and *Klebsiella pneumoniae* SM6 and SM1 also showed the phosphate solubilisation ability by producing organic acids (Rajput et al., 2015). This is probably the first report on phosphate solubilising capability of *C. davisae*.

Organic phosphate in soil is mainly present as phytic acid and calcium or sodium phytate that are unavailable to plants and therefore, it becomes necessary to determine rhizospheric bacteria with the capability of phytate hydrolysis (Alori et al., 2017). The phophatase family enzyme phytase (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8) catalyses the hydrolysis of phytate (myo-inositol hexakisphosphate) to myo-inositol and soluble Pi. Among the isolated microorganisms RS3, RS23, R26 and RS49 produced extracellular phytase with substantial activity in the pH range 4.5–7.5 indicating their ability to hydrolyze phytate in soil with similar pH values. A wide group of phytase-producing bacteria have already been reported, including *Bacillus subtilis* (Keruvuo et al., 1998; Kumar et al., 2012; Reddy et al., 2015), *Bacillus* sp. (Kim et al., 1998; Choi et al., 1999; Choi et al., 2001), *Enterobacter* sp. (Yamata et al., 1968; Yoon et al., 1996; Kim et al., 2003), *Pseudomonas* sp. (Richardson and Hadobas, 1997), *Advenella* sp. (Singh et al., 2014), *Bacillus* sp. (Zaheer et al., 2019).

Zinc is an essential element for optimum plant growth. The plant Zn deficiency leads to reduced carbohydrates, auxins, nucleotides, cytochromes, and chlorophyll synthesis; reduced integrity of membrane, and increased susceptibility to high temperature (Singh et al., 2005). Bacterial isolates capable of solubilizing Zn in soil are considered as potential alternates to Zn fertilization. There are several mechanisms for zinc solubilisation that are used by microorganisms, one of which is acidification. Microbes sequester the soil zinc cations through the production of organic acid and reduce the pH of the nearby soil (Alexander, 1997). Production of siderophores and oxidoreductive systems on cellular membrane, and chelating ligands are the other mechanisms for zinc solubilisation used by

microbes (Wakatsuki, 1995; Chang et al., 2005; Saravanan et al., 2011). Present study found some PGPR belonging to genera *Klebsiella*, *Cedecea*, *Bacillus*, *Burkholderia* and *Acinetobacter* as Zn solubilizers with highest ZSI value for *Cedecea* species. Our findings are consistent with previously reported literature where numerous PGPR have shown to improve the plant growth and intracellular zinc content at agriculture scale, which include *Bacillus aryabhattai* (Ramesh et al., 2014), *Bacillus* sp. and *Azospirillum* (Hussain et al., 2015), *Pseudomonas*, Rhizobium strains (Deepak et al., 2013; Naz et al., 2016). In laboratory scale numerous PGPR strains have been reported to solubilise Zn, including *Burkholderia cenocepacia* (Pawar et al., 2015), *Bacillus* sp., *Pseudomonas striata*, *P. fluorescence*, *Klebsiella pneumoniae*, *Serratia liquefaciens*, *S. marcescens*, and *Bacillus thuringiensis* (Ullah et al., 2015), *Cedecea davisae*, *P. aeruginosa* (Fasim et al., 2002), *Gluconacetobacter diazotrophicus* (Saravanan et al., 2007).

Due to its ability to enhance root development and improve mineral uptake, IAA is considered as an important PGP trait (Santoyo et al., 2016). The rhizobacteria mainly use tryptophan as precursor for IAA synthesis, however tryptophan independent pathways have also been described in a report (Ribeiro and Cardoso, 2012), which explains the synthesis of IAA in absence of tryptophan by soil rhizobacteria. Greater than 80% of IAA producing soil bacteria colonize plant root surface and in conjunction with plant endogenous IAA stimulate the root system to increase size, increase number of adventitious roots and making them enable to up take more nutrient from soil for plant growth (Patten and Glick, 2002). Out of various PGPR isolated from mustard rhizosphere few genera of *Pseudomonas*, *Rhizobium*, *Cedecea*, *Azotobacter*, *Acinetobacter*, *Klebsiella*, *Burkholderia* and *Bacillus*, produced IAA. Moreover, within the IAA producers *Klebsiella pneumoniae* RS26 was found to produce highest amount of IAA both in presence and absence of tryptophan, which were, 14.51 ± 1.1 and 7.13 ± 0.94 µg mL^{-1}, respectively. Another strain, *Cedecea* RS3 also showed significant amount of IAA production and recorded yield in presence and absence of tryptophan were 10.13 ± 1.01 and 5.63 ± 0.89 µg mL^{-1}, respectively. IAA biosynthesis has previously been reported for various N-fixing bacteria that include *Azospirillum* sp., *Bacillus* sp., *Gluconacetobacter* sp., *Burkholderia* sp., *Paenibacillus* sp., *Sphingomonas* sp., *Herbaspirillum* sp., and *Pseudomonas* sp (Dobbelaere et al., 2003, Islam et al., 2009). It has been reported that phytohormones produced by rhizobacteria are more effective by virtue of their continuous and slow release (Mohite, 2013).

The role of rhizobacteria in modulating ethylene phytohormone synthesis has been widely studied. Many soil bacteria are known to produce the ACC deaminase that degrade ACC, the direct precursor of ethylene, into ammonia and α-ketobutyrate and thus impart a positive effect on stimulating root and plant growth (Glick et al., 2007; Franche et al., 2009). In this study, a wide range of N-fixing PGPR, such as *Bacillus*, *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Cedecea* and *Azotobacter* exhibited ACC deaminase activity. Similar results were also observed by Glick (2012), wherein they found the production of ACC deaminase by *Bacillus* sp., *Pseudomonas* sp., *Klebsiella* sp., *Cedecea* sp., *Burkholderia* sp. and *Serratia* sp.

The proper selection of potent microorganisms is a kind of risk-benefit analysis which requires a study at strain level to analyse the outputs of each isolates and select the most potent strains. At this point, in our study a multivariate analysis (Principal component analysis; PCA) was performed as a tool to achieve insight into the complexity of the 11 isolates (those showing at least 4 PGP traits) quantitative PGP traits to reduce their number and to select the most potent strains. There are many techniques and approaches for data clustering and classification such as k-means, principal component analysis, cluster analysis and multiple correspondences and which have their own benefits and limitations (Di Benedetto et al., 2019). However, PCA is the most appropriate and suitable approach because (i) PCA reduces many variables to a smaller number, while losing very little information as possible (reduction of the complexity), (ii) PCA can segregate the samples into stratum or homogenous group (clustering) and (iii) PCA can gives an overview of the important variables which perform a crucial role in clustering (leading variables). Therefore, in this study PCA was employed for selection of bacterial strains for plant application. Two microbial strains RS3 and RS26 were found to have significant, positive, contributions to most PGP attributes and selected for the pot trial experiment for improving the mustard plant growth. A summary of the strategy for selection of PGPR is depicted in Fig 2.17.

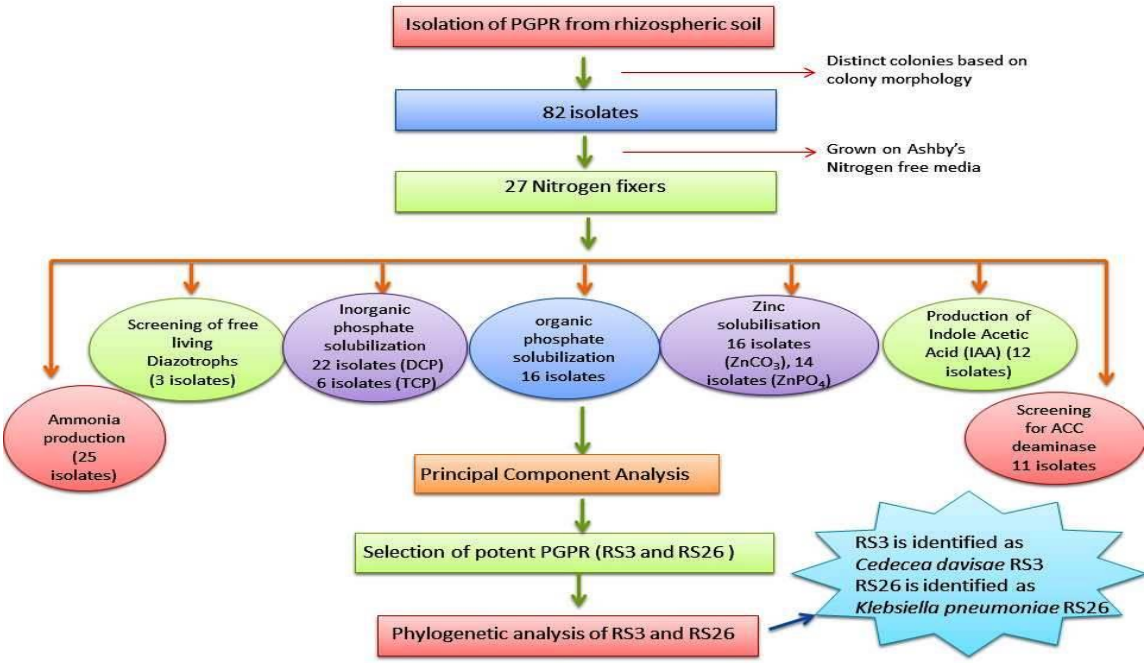


Fig. 2.17. Flow diagram of the isolation, screening and identification of PGPR isolates

In conclusion, all the 27 N_2-fixing strains were evaluated for multiple PGP traits, including, ammonia production, phosphate solubilisation, phytase production, zinc solubilisation, IAA production and ACC deaminase production. Most of the bacterial strains tested possessed at least 4 or more PGP traits and *C. davisae* RS3 and *K. pneumoniae* RS26 were selected as the two most potent PGPR strains using PCA of their PGP traits.

Supplementary Table: **Details of PGPR with shared PGP attributes as shown by the in venn-diagram**			
Figure No.	**PGP attributes shared**	**No. of isolates**	**Name of the isolates sharing the PGP attributes**
2.5 a	N_2 fixation, IAA production, ACC deaminase production	3	RS3, RS10, RS26
2.5 a	N_2 fixation, IAA production	9	RS7, RS9, RS11, RS23, RS31, RS46, RS48, RS49, RS51
2.5 b	N_2 fixation, DCP solubilization, TCP solubilization	2	RS3, RS7
2.5 b	N_2 fixation, DCP solubilization, TCP solubilization, OP solubilization	2	RS3, RS20
2.6 a	N_2 fixation, ZC solubilization, ZP solubilization	7	RS2, RS3, RS9, RS14, RS26, RS61, RS74
2.6 a	N_2 fixation, ZC solubilization	9	RS6, RS23, RS39, RS46, RS48, RS49, RS53, RS65, RS77
2.6 b	ZC solubilization, ZP solubilization, DCP solubilization, TCP solubilization, OP solubilization	1	RS3
2.6 b	ZC solubilization, ZP solubilization, OP solubilization	3	RS3, RS14, RS26
2.7 a	IAA production, ACC deaminase production, DCP solubilzation,	3	RS3, RS10, RS31
2.7 a	IAA production, ACC deaminase production, DCP solubilzation, OP solubilisation	1	RS3
2.7 b	IAA production, ACC deaminase production, ZC solubilization, ZP solubilization	1	RS3
2.7 b	IAA production, ACC deaminase production, ZP solubilization	1	RS10

CHAPTER THREE

APPLICATION OF POTENT PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) FOR GROWTH ENHANCEMENT OF MUSTARD (*Brassica campestris* L.)

3.1. Introduction

Intensive agricultural processes aiming for high yield and quality crops, need massive use of chemical fertilizers, which are not only costly but also environmentally unfavourable creating air, water and soil pollutions. Hence, the eco-friendly, organic and sustainable agriculture processes are highly desirable (Esitken et al., 2005). The organic agricultural practices mainly focus on biofertilizers based on PGPR, crop residues and animal manures and the use of synthetic chemical fertilizers, pesticides, synthetic hormones or growth regulators etc. are avoided. However, the major problem associated with organic farming is the reduced crop yield (Bengtsson et al., 2005). PGPR with the ability to solubilize insoluble soil nutrients, produce plant growth regulators and induce plant defence system against pathogens, promote plant growth along with the maintenance of sustainable environment and soil health (O'Connell, 1992; Babalola and Glick, 2012; Ahemad and Kibret, 2014). Several bacterial genera such as *Bacillus*, *Klebsiella*, *Arthrobacter*, *Azospirillium*, *Azotobacter*, *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Serratia* etc. have been reported as important PGPR strains (Sturz and Nowak, 2000; Sudhakar et al., 2000; Dinesh et al., 2015; Gouda et al., 2018; Sapre et al., 2018, Akinrinlola et al., 2018). Several scientific reports depicted the beneficial effect of PGPR on growth and yield promotion of apple, citrus, high bush blueberry, mulberry, banana, carrot, lettuce, cabbage, soybean, chickpea, wheat, maize (Kloepper, 1994; De Silva et al., 2000; Sudhakar et al., 2000; Esitken et al., 2002, 2003; Pirlak et al., 2007; Miransari et al., 2016; Rai and Nabti, 2017; Marinković et al., 2018; Khan et al., 2018; Wang et al., 2020; Mishra and Chauhan, 2020).

Plant productivity has fundamental dependence on N-fertilizers, mainly in the form of ammonium (NH_4^+) and nitrate (NO_3^-). However, the crop plants cannot take up N-fertilizers efficiently and 50-70% of applied N is lost from the plant-soil system (Gouda et al., 2018). Excess N in soil has deleterious effect on the quality of air, water, and soil, with life threatening consequences. Hence, it is absolutely necessary to improve the N-fertilizer use efficiency of the crop plants in order to reduce both the cost associated with the crop production and environmental

damage (Diaz and Rosenberg, 2008; Xu et al., 2012). Biological nitrogen fixation (BNF) activity is one of the most important plant growth promotion traits of PGPR. BNF involves the reductive conversation of atmospheric N_2 to plant utilizable form NH_3, which is mediated in nature by bacteria and some species of actinomycetes (Baldani et al., 2002). Therefore, the N-fixing PGPR (known as diazotrophs) are widely applied in agriculture as an alternative to the chemical N-fertilizers (Meunchang et al., 2004; Sofi and Wani, 2007; Woyessa and Assefa, 2011). It has been found that NH_3 produced by BNF act as a signal molecule to the plant root complex molecular network and can regulate the expression of thousands of plant genes which are involved in N uptake and plant growth and development (Li et al., 2012). Although N-fixing PGPR have shown to enhance plant productivity, their role on the improved utilization of inorganic N-fertilizer are limiting.

Mustard (*Brassica campestris* L.) is a rabi crop belonging to the family Cruciferae. It is the second most important edible oilseed crop in India, after groundnut and accounts for nearly 30% of the total oilseeds produced in the country. The mustard growing regions in India are experiencing vast diversity in the agro-climatic conditions (Shekhawat et al., 2012). Under marginal resource situation, cultivation of mustard becomes less remunerative to the farmers. This also results in a big gap between requirement and production of mustard. Therefore, plant nutrition management has to be improved upon the existing yield levels obtained at farmers field.

Response surface methodology (RSM) approach is suitable for multifactor experimental design because it takes into consideration the correlation effects between multiple factors. The empirical statistical method using RSM is generally applied to evaluate the model generated multiple regression equation and hence, can determine the optimum values of each of the independent factor to maximize the response variable (Chakravarti and Sahai, 2002; Razak et al., 2015).

The previous chapter described the isolation of PGPR from chickpea rhizosphere followed by the selection of two potent PGPR, RS3 and RS26 on the basis of qualitative and quantitative analysis of plant growth promotion (PGP) traits. Based on 16S rRNA gene analysis RS3 and RS26 were characterized as *Cedecea davisae* and *Klebsiella pneumoniae* and therefore, named as *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26, respectively. In the present study the two PGPR were supplemented into the nutrient formulations (NFs) with varying N levels and their effects on growth and productivity of mustard plant were determined. Plants were treated with the NF using a general and RSM optimized approaches, and their effects on mustard plant growth and yield were compared and analyzed by statistical tools. Further transcriptomics analysis of the plants treated with RSM optimized NF and N-appropriate NF was performed in order to determine the differential effect PGPR supplementation to NF at transcriptional level.

3.2. Materials and Methods

3.2.1. Plant materials, Strains, Chemical and reagents

Mustard plant (*Brassica campestris* cv. B_9) seeds were obtained from the Department of Plant Breeding, Uttar Banga Krishi Visvavidyalaya, Coochbehar, West Bengal. The bacterial strain *Cedecea davisae* RS3 (Gene Bank accession number KX101223) and *Klebsiella pneumoniae* RS26 (Gene Bank accession number MH819506.1) were isolated from the rhizospheric soil from the agriculture field near University of North Bengal, as mentioned in Chapter 2. All chemicals, biochemical and microbiological media used in this study were purchased from Himedia, India; Sigma-Aldrich, USA; and E. Merck, Germany.

3.2.2. Soil collection and analysis

Surface soil was collected from the non-fertilized field site, near University of North Bengal, West Bengal, India. The physico-chemical properties of the soil which are as follows: clay-18.7%, slit-26.3%, sand-54.1%, texture-Sandy Loam, pH-6, EC,0.13 ds m^{-1}; Nitrogen,1.3 g kg^{-1}; Carbon, 15 g kg^{-1}; Potassium, 60 mg kg^{-1}; Phosphorus, 25 mg kg^{-1}; Sulfur, 35 mg kg^{-1}; Ca^{2+}, 0.24 mol kg^{-1}, Mg^{2+}, 0.94 mol kg^{-1}.

3.2.3. Preparation of soil and planting material for pot experiments

Soil was sieved through a mesh of approximately 4 mm size to remove rocks, clods and large pieces of organic matter and then autoclaved to reduce the load of existing microbes. About 2kg of soil is filled into each high density polyethylene pots (20 x 25cm) with drainage (16 holes of 5mm diameter).

Seeds of mustard (*Brassica campestris* L.) and were surface disinfected by soaking in 1 % (w/v) sodium hypochlorite for 10 min and then washing thoroughly with sterile distilled water. Disinfected seeds were kept on the surface of moist filter paper in an incubator at 25ºC for 2-3 days under dark for germination. Germinated seedlings of almost equal size were planted into the pots (4 seedlings/pot) filled with sterilized soil.

3.2.4. Determination of growth characteristic of *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26 and preparation of inocula for plant application

The growth curve of the bacterial strains was determined by turbidometric method. The bacterial culture was individually inoculated into 250 mL of nutrient broth and incubated at 37 ºC with 120 rpm shaking. Aliquot (1 mL) was withdrawn at 30 min intervals and its optical density was monitored at 610 nm, until the stationary phase was observed (Fig.3.1).

For preparation of inocula, each bacterial isolate was grown separately on 250 mL of nutrient broth and the mid exponential phase cultures was centrifuged at 5000 rpm for 10 min at 4 ºC. The bacterial pellet was suspended in sterilized NF and the absorbance at 610 nm (A_{610}) was adjusted

to 0.6 (10^7 CFU mL^{-1}). For the preparation of NF by general method, the nutrient solution was supplemented with 10 % (v/v) each of the two strains. For preparation of RSM optimized NF, the volume of both of the strains (% v/v) were kept as per the CCRD experimental design mentioned in Table 3.1.

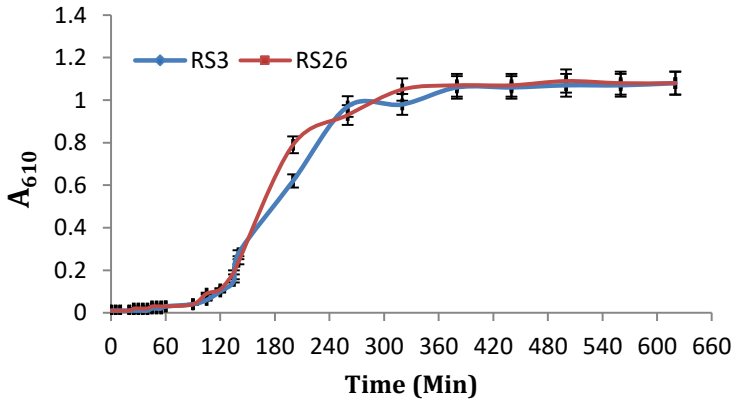


Fig 3.1: Growth curves of *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26

3.2.5. Antagonistic activity assay

Antagonistic activity of RS3 and RS26 isolates was performed by modified cross-streak method (MCSM), as mentioned by Velho-Pereira and Kamat (2011). A loopful culture of RS3 was singly streaked at the centre of the Mueller-Hinton agar (MHA) plate and incubated at 37 ^oC for 24 h and then RS26 was streaked perpendicular to the previous streak of RS3. The plate was kept at 37 ^oC for further 24h and observed for the presence of zone of inhibition. The protocol was repeated vice-versa with initial streaking with RS26 followed by that of RS3.In another MHA plate, streaks of both the strains RS3 and RS26 were done perpendicular to each other and then incubated for 24 h at 37^oC to check the inhibition zone.

3.2.6. Hemolysin production assay

Sheep blood agar plate was prepared by adding 5 % sheep blood in autoclaved Trypticase soy agar (Cafiso et al., 2012). The isolated PGPR strains RS3, RS26 and a hemolysin positive bacterial strain (control) were point inoculated into sheep blood agar plate and incubated at 37 ^oC for 48 h.

3.2.7. Effect of PGPR on growth of mustard plant

3.2.7.1. Preparation of NFs by general method and plant application

The physical conditions required for the pot experiment in a greenhouse was maintained at 25 ± 2^oC, 60-70 % relative humidity, and 13 h dark and 11 h light period. The pot experiment was carried out in triplicate for 3 months. Four germinated seedlings were transferred to each pot and after seven days of growth pots were divided into four different treatment groups with nine pots in

each group, namely, N-appropriate without microbes (N^+PGPR^-), N-appropriate with microbes (N^+PGPR^+), N-deficit without microbes (N^-PGPR^-), N-deficit with microbes (N^-PGPR^+). All the four groups of plants were irrigated twice a week with 50 mL of their respective NF as described below:

A. N-appropriate without microbes (N^+PGPR^-)

The N^+PGPR^- treatment group plants were irrigated with NF containing $(NH_4)_2SO_4$, 5 mM; NaH_2PO_4, 0.3 mM; K_2SO_4, 0.5 mM; $CaCl_2$, 1 mM; $MgSO_4$, 1.6 mM; Fe-EDTA, 0.05 mM; $Na_2MoO_4.2H_2O$, 0.06 µM; H_3BO_3, 0.015 mM; $MnCl_2$, 0.008 mM; $ZnSO_4$, 0.12 µM, and $FeCl_3$, 0.029 mM.

B. N-appropriate with microbes (N^+PGPR^+)

The plants of this group were treated with NF mentioned above for N^+PGPR^- supplemented with 10% (v/v) each of RS3 and RS26. The bacterial inoculum was prepared by the method described in section 3.2.4.

C. N-deficit without microbes (N^-PGPR^-)

For this treatment group the N level of the NF mentioned above for N^+PGPR^- group was reduced to 0.5 mM.

D. N-deficit with microbes (N^-PGPR^+)

For this treatment regime, NF mentioned above for N^-PGPR^- was supplemented with 10% (v/v) each of RS3 and RS26 isolates. The bacterial inoculum was prepared by the method described in section 3.2.4.

Plants were harvested from separate pots of all the above treatment groups at 15, 45 and 75 days after treatment (DAT) and were analyzed for various morphological and biochemical parameters. Plant samples were also preserved by freezing in liquid nitrogen and stored for further use.

3.2.7.1.1. Determination of root and shoot length

The plants were carefully uprooted without affecting the roots at 15, 45 and 75 DAT. The length of the plant shoot and root were measured using a centimeter scale.

3.2.7.1.2. Determination of fresh and dry weight of root and shoot

The harvested plants were carefully washed to prevent root loss and blotted off to remove excess surface water. Fresh weight (FW) of root and shoot were measured on a scale of ±0.001g at an ambient temperature 25^oC. After that, the root and shoot samples were dried in convection oven at 80 ^oC for 48 h and then weighed to determine the dry weight in the same scale of ±0.001g.

3.2.7.1.3. Determination of number of branches and siliqua per plant, number of seeds per siliqua, 100 seed weight and seed yield

Plants grown upto the maturation stage (75 DAT) was used for measuring the number of branches and number of siliqua per plant, by manual counting. Number of seeds per siliqua was determined by carefully opening the siliqua followed by counting them manually. Weight of 100 seeds was also determined.

3.2.7.1.4. Determination of chlorophyll a and chlorophyll b contents

1g of leaflet tissue was extracted in 10 mL of 80% (v/v) chilled acetone followed by centrifugation at 9500 RPM for 10 min at 4ºC. The absorbance of clarified supernatant was measured spectrophotometrically at 663 nm and 645 nm. The chlorophyll b and chlorophyll a contents were determined according to the Equation 1 and 2 (Arnon, 1949). The chlorophyll content was expressed as mg per gram FW (mg g^{-1} FW) of the leaf.

Chlorophyll a (mg g^{-1}) = (12.7 X A_{663}) − (2.59 X A_{645}) …… (Eq.1)

Chlorophyll b (mg g^{-1}) = (22.9 X A_{645}) − (4.7 X A_{663}) ……... (Eq.2)

3.2.7.1.5. Determination of root and shoot protein contents

The harvested plants were washed with running tap water followed by distilled water. 1 g of root or shoot was treated with liquid N_2 and then homogenized in 5 mL of 0.1 M phosphate buffer pH 7, using mortar and pestle. After filtering through muslin cloth, the homogenate was centrifuged at 10,000 RPM at 4 ºC for 15 min. The protein content of the clear supernatant was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) (0-100 µg mL^{-1}) as standard (Fig. 3.2). To 100 µl of sample, 3 mL of Bradford reagent [100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL 95 % ethanol; to the solution 100 mL 85 % (w/v) phosphoric acid was added and total volume was made 1 L with distilled water] was added and mixed thoroughly by vortexing followed by incubation at room temperature for 10 min. The absorbance of the resulting mixture was recorded at 595 nm. Protein concentration was calculated from a standard curve using 0-100 µg L^{-1} BSA (Fig. 3.2). The assay was done in triplicates. Amount of protein expressed as mg g^{-1} FW.

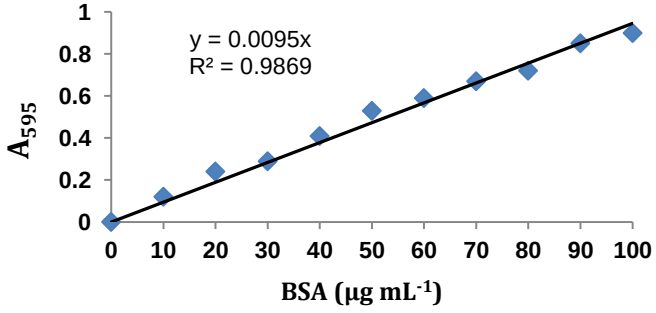


Fig 3.2: Standard curve of bovine serum albumin

3.2.7.1.6. Determination of root and shoot carbohydrate contents

Plants harvested at 15, 45 and 75 DAT from individual pots were washed with running tap water followed by distilled water. 1 g of root or shoot was treated with liquid N_2 and then extracted with 5 mL of 0.1 M phosphate buffer pH7, using mortar and pestle. The extract was filtered through muslin cloth followed by centrifugation at 10,000 rpm at 4 ^oC for 15 min. The clear supernatant was used for estimation of total carbohydrate by Anthrone method using glucose as standard (Yemm and Willis, 1954). To 1 mL of the supernatant 4 mL of Anthrone reagent was added [200 mg of anthrone dissolved in 100 mL of ice cold 95% H_2SO_4] and the mixture was heated in boiling water bath for 10 min followed by rapid cooling. The absorbance of the resulting mixture was recorded at 630 nm. Carbohydrate concentration was calculated from a standard curve using 0-1000 µg L^{-1} glucose (Fig. 3.3). The assay was done in triplicates. Amount of carbohydrate expressed as mg g^{-1} FW.

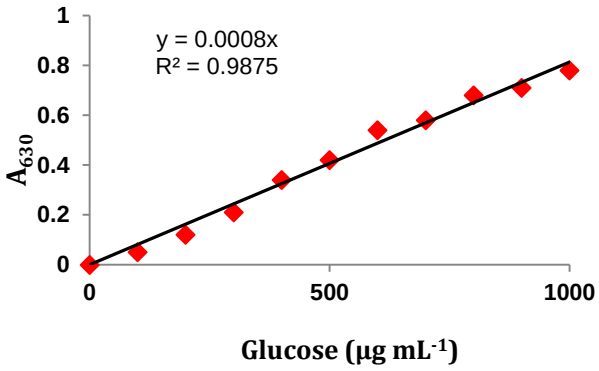


Fig 3.3: Standard curve of glucose

3.2.7.1.7. Determination of glutamine synthetase (GS) activity of root and shoot

The plant root or shoot tissue was extracted with extraction buffer [containing 100mM Tris-HCl (pH 7), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15% glycerol (5 mL g^{-1} FW)] using mortar and pestle. The extract was filtered through muslin cloth followed by centrifugation at 10,000 rpm at 4 ^oC for 15 min. Glutamine synthetase (GS) activity of the supernatant was estimated by semi-synthetase reaction as described by Singh and Ghosh (2013). The reaction mixture (final volume 1mL) contained 25 mM Tris-HCl (pH 7), 200 mM glutamate, 10 mM ATP, 5 mM hydroxylamine hydrochloride, 20 mM $MgCl_2$ and 100 µl of the clear supernatant as enzyme source. The reaction mixture was incubated at 37 ^oC for 30 min. Thereafter, the reaction was terminated after by adding 2 mL of $FeCl_3$ reagent [0.67 M $FeCl_3$, 0.37 M HCl and 20 % (w/v) trichloroacetic acid]. Reaction terminated at 0 min incubation served as blank. After 20 min the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm γ-glutamylhydroxamate concentration was determined from a reference

curve prepared with 0 - 2 µmole γ-glutamylhydroxamate (Fig 3.4). One unit of GS activity was defined as the formation of 1 µmole of γ-glutamylhydroxamate produced min^{-1}.

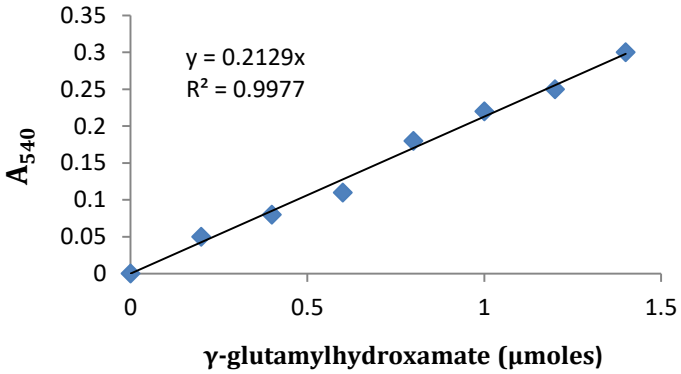


Fig. 3.4: Standard curve of γ-glutamylhydroxamate

3.2.7.2. Effect of PGPR on the plants using RSM approach

Three variables Central Composite Rotatable Design (CCRD) combined with RSM (using Design Expert software) was carried out to optimize the NF treatment. The methods for planting of mustard seedlings and generation of bacterial inoculum were same as described in Section 3.2.3 and 3.2.4. The optimization was based on the study of interaction among three effective parameters i.e. N concentration (mM), inoculum volume of RS3 (%) and inoculum volume of RS26 (%). For allowing the curvature in the model, CCRD was employed in RSM having "axial points" around the centre point. The distance of each factor from its centre points to its factorial point is ±1, whereas the axial points of each factor differ from its centre point by ±α and α is related to the number of independent variables by the following equations:

$\alpha = (2k)^{1/4}$……..(Eq.1)

Where k is the number of variables used to construct the model.
The three independent variables employed to generate the models were N concentration in the formulation (A), inoculum volume of strain 1 i.e. *C. davisae* RS3 (B) and inoculum volume of strain 2 i.e. *K. pneumoniae* RS26 (C), with their five different coded levels [-α, -1, 0, +1, +α]. The actual values of the coded variables are presented in Table 1. The coded and actual values of the input variables are related by the following equations:

$X_a = (Z_a - Z_0)/\Delta Z$…….....(Eq. 2)

Where, X_a and Z_a represent the coded and actual values of input variables, respectively, Z_0 is the actual value of the variables at its centre point and ΔZ is the step change value of the same factor.

In total, 20 experimental runs were selected and each experiment was conducted in triplicate based on Eq. 3

$R= 2^k+2k+n_0$……..... (Eq. 3)

Where, R is the total number of experimental runs, k represents the number of independent factors employed to construct the model and n_0 is the number of repetitions in the centre point. The total 20 experimental runs were composed of 8 factorial run (2^k), 6 axial run (2k) and 6 repetitions (n_0) around the centre point. The predicted response variables (Y), such as carbohydrate content of shoot (mg g^{-1} FW), proteins content of shoot (mg g^{-1}FW) and seed yield (g $plant^{-1}$) were analyzed by the following second order polynomial regression Eq. 4:

$Y=a_0+a_1x_1+a_2x_2+a_3x_3+a_{11}x_{12}+a_{22}x_2^2+a_{33}x_3^2+a_{12}x_1x_2+a_{13}x_1x_3+a_{23}x_2x_3$……..(Eq.4)

Where, Y is the predicted response variables, a_0 is the intercept terms, x_i is the independent factor coded/actual terms and a_i is the model coefficient terms.

50 mL of NF containing specified percentage of inocula were applied to plants twice a week. Sampling was done from individual pots during maturation stage at 75 DAT and assayed for three response variables, namely carbohydrate content of shoot (mg g^{-1} FW), protein content of shoot (mg g^{-1} FW) and seed yield (g $plant^{-1}$), as indices of growth and yield.

Table 3.1
Design parameters in coded and actual terms

Factor codes	Factor	-α	-1	0	+1	+α
A	N Concentration (mM)	-1.03/0	0.50	2.75	5	6.53
B	Strain 1 (% v/v) (RS3)	-3.64/0	10	30	50	63.64
C	Strain 2 (% v/v) (RS26)	-3.64/0	10	30	50	63.64

3.2.8. Transcriptomic analysis of mustard plants

Mustard plant treated with N appropriate (5 mM N) NF without PGPR (designated as control) and plants treated with RSM optimized condition (0.5 mM nitrogen) along with PGPR (designated as treated) were harvested at 75 DAT and roots of these two groups of plants were used for transcriptomic analysis.

3.2.8.1. Sample preparation and RNA extraction

Root samples were collected from plants of the two different treatment groups viz. N appropriate NF without PGPR (N^+PGPR^-) and plants treated with RSM optimized condition (with PGPR).

Root samples were washed carefully with distilled water, blotted dry and immediately frozen in liquid-N_2 for RNA extraction. Total RNA was isolated from the root sample using ZR plant RNA mini preparation (ZYMO research) according to the manufacture's instruction. The quality and quantity of the extracted RNA was checked on 1% denaturing RNA agarose gel and nano drop technique, respectively.

3.2.8.2. Preparation of cDNA library and transcriptome sequencing

The RNA sequence paired end sequencing libraries were prepared from the RNA samples using IlluminaTruSeq standard mRNA sample preparation kit. Briefly, mRNA was enriched from the total RNA using poly-T attached magnetic beads, followed by enzymatic fragmentation, 1st strand cDNA conversion using Duper script II and Act-D mix to facilitate RNA dependent synthesis. The 1st strand cDNA was then synthesized to second strand using second strand mix. The double stranded cDNA was then purified using AMPure XP beads followed by A-tailing, adapter ligation and then enriched by limited number of PCR cycles. The PCR enriched libraries were analyzed on 4200 Tape Station System (Agilent Technologies) using high sensitivity D1000 screen tape as per manufacturer's instructions. After obtaining the Qubit concentration for the libraries and the mean peak sizes from Agilent Tape station profile, the PE Illumine libraries were loaded onto NextSeq500 for cluster generation and sequencing. Paired-end sequencing allows the template fragments to be sequenced in both the forward and reverse direction on NextSeq500.

3.3. Result

3.3.1. Determination of compatibility of PGPR strains

Initially, the compatibility of PGPR strains, *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26, was determined by antagonistic assay using modified cross streak method. The results in Fig. 3.5 show absence of growth inhibition zone indicating compatibility between the two organisms and therefore, can be applied together for pot experiments.

3.3.2. Determination of pathogenicity of PGPR strains

The pathogenicity potential of PGPR strains, *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26 was determined by hemolysin assay. Both the strains didn't show any clear halo zone on blood agar plates indicating their non-pathogenic nature and therefore, were suitable for present study (Fig 3.6).

3.3.3. Effect of PGPR on the mustard plants treated with NF using general approach

The results in Table 3.2 show that during all the three stages of growth, the root FW and length were significantly higher for plants belonging to $N^{-}PGPR^{+}$ treatment group. Consequently, the average root lengths of 1.89, 4.36 and 6.76 cm were recorded at 15 DAT, 45 DAT and 75 DAT, respectively, which were significantly ($p<0.05$) higher as compared with other treatment groups. At 75 DAT, the root and shoot carbohydrate content of $N^{-}PGPR^{+}$ plants were 6.76 and 12.17 mg g^{-}

^{1} FW, respectively, and that of $N^{+}PGPR^{+}$ plants were 5.42 and 11.32 mg g^{-1} FW, respectively (Table 3.3). The protein content of root (378.67 mg g^{-1} FW) and shoot (675.89 mg g^{-1} FW) was also significantly higher for $N^{-}PGPR^{+}$ plants (Table 3.3). Similarly, the seed yield parameter, like number of siliqua per plant (39.40), number of seeds per siliqua (30.12), 100 seed weight (0.48 g) and seed yield (5.76 g $plant^{-1}$) at 75 DAT were maximum in $N^{-}PGPR^{+}$ treatment group (Table 3.4). Though $N^{-}PGPR^{+}$ treated plants had lesser number of branches (4.23) than $N^{+}PGPR^{+}$ plants, they had more number of siliqua in the branches. From the results, it can be concluded that among the three general treatments, $N^{-}PGPR^{+}$ treatment was optimum for enhanced growth and seed yield of the mustard plant.

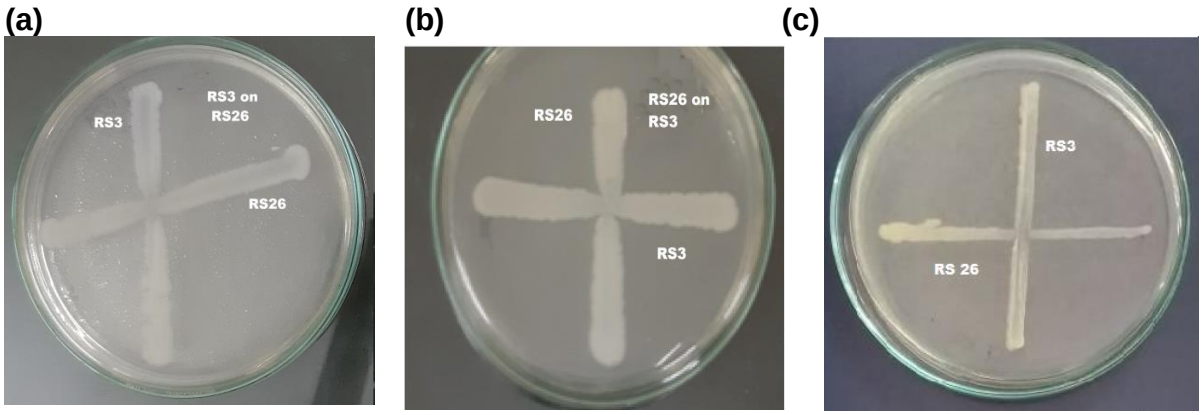


Fig 3.5. Antagonistic activity of RS3 and RS26 (a) perpendicular streak of RS3 on 24 h old RS26 streak (b) perpendicular streak of RS26 on 24 h old RS3 streak (b) simultaneous cross streak of RS3 and RS26

Fig 3.6: Pathogenicity test for RS3, RS26 and positive control

Table 3.2
Effect of PGPR on the plant morphological characteristics in response to different nitrogen treatment conditions. Mean values marked with different alphabets are significantly different at $p<0.001$, as suggested by one way analysis of variance

Treatment Group	Root FW(g)	Root dry weight (g)	Shoot FW (g)	Shoot dry weight (g)	Root length (cm)	Stem length (cm)
N^-PGPR^+ (15 DAT)	0.43^{b}	0.11^{b}	0.76^{bc}	0.31^{c}	1.89^{b}	8.56^{d}
N^-PGPR^+(45 DAT)	1.08^{f}	0.67^{de}	1.43^{g}	0.92^{h}	4.36^{e}	14.87^{i}
N^-PGPR^+(75 DAT)	1.17^{g}	0.78^{e}	1.56^{h}	1.16^{i}	6.76^{h}	20.12^{jk}
N^-PGPR^- (15 DAT)	0.36^{a}	0.06^{a}	0.43^{a}	0.09^{a}	1.02^{a}	4.54^{a}
N^-PGPR^- (45DAT)	0.87^{d}	0.42^{c}	0.89^{c}	0.41^{d}	3.78^{d}	9.67^{e}
N^-PGPR^- (75DAT)	1.05^{f}	0.57^{d}	1.21^{e}	0.79^{f}	4.37^{e}	11.32^{g}
N^+PGPR^+15DAT)	0.43^{b}	0.12^{b}	0.67^{b}	0.26^{b}	1.78^{b}	6.45^{c}
N^+PGPR^+(45 DAT)	0.89^{d}	0.54^{d}	1.32^{f}	0.89^{g}	3.98^{d}	10.65^{f}
N^+PGPR^+(75 DAT)	1.07^{f}	0.62^{de}	$1.\ 43^{g}$	0.97^{h}	5.76^{g}	15.78^{ij}
N^+ $PGPR^-$ (15 DAT)	0.42^{b}	0.09^{a}	0.65^{b}	0.23^{b}	1.76^{b}	5.09^{b}
N^+ $PGPR^-$ (45 DAT)	0.79^{d}	0.38^{c}	1.10^{d}	0.68^{e}	3.54^{d}	10.12^{f}
N^+ $PGPR^-$ (75DAT)	0.93^{e}	0.58^{d}	1.32^{f}	0.87^{g}	4.87^{f}	12.45^{h}

Table 3.3
Effect of PGPR on the plant carbohydrate and protein content in response to different nitrogen treatment conditions. Mean values marked with different alphabets in each column are significantly different at $p<0.001$, as suggested by one way analysis of variance

Treatment Group	Root Carbohydrate (mg g^{-1} FW)	Shoot Carbohydrate (mg g^{-1} FW)	Root Protein (mg g^{-1} FW)	Shoot Protein (mg g^{-1} FW)
N^-PGPR^+ (15 DAT)	2.12^{c}	6.34^{c}	96.17^{c}	235.32^{e}
N^-PGPR^+ (45 DAT)	4.34^{e}	8.67^{e}	234.37^{e}	543.72^{h}
N^-PGPR^+ (75 DAT)	6.76^{g}	12.17^{h}	378.67^{f}	675.89^{i}
N^- $PGPR^-$ (15 DAT)	0.87^{a}	4.33^{a}	26.78^{a}	67.68^{a}
N^- $PGPR^-$ (45DAT)	2.45^{c}	7.23^{d}	98.09^{c}	128.56^{c}
N^-PGPR^- (75DAT)	3.06^{d}	9.78^{f}	138.12^{d}	197.98^{d}
N^+PGPR^+ (15DAT)	1.34^{b}	5.14^{b}	68.89^{b}	126.43^{c}
N^+PGPR^+ (45 DAT)	3.45^{d}	7.18^{d}	159.87^{de}	325.23^{f}
N^+PGPR^+ (75 DAT)	5.42^{f}	11.32^{g}	267.34^{e}	454.83^{g}
N^+ $PGPR^-$ (15 DAT)	1.37^{b}	4.36^{a}	43.36^{bc}	98.96^{b}
N^+ $PGPR^-$ (45 DAT)	2.48^{c}	6.36^{c}	96.78^{c}	178.12^{c}
N^+ $PGPR^-$ (75DAT)	4.32^{e}	9.78^{f}	157.98^{d}	265.67^{e}

Table 3.4
Effect of PGPR on the plant seed yield in response to different treatment conditions
Mean values marked with different alphabets in each column are significantly different at p<0.001, as suggested by one-way analysis of variance. 'na'=not available

Treatment Group	No. of branches	No.of Siliqua/plant	Seed/siliqua	100 seed weight (g)	Seed yield/plant (g)
$N^{-}PGPR^{+}$ (15 DAT)	1.21^{a}	Na	na	na	na
$N^{-}PGPR^{+}$ (45 DAT)	2.65^{d}	Na	na	na	na
$N^{-}PGPR^{+}$ (75 DAT)	4.23^{f}	39.40^{d}	30.12^{d}	0.48^{c}	5.76^{d}
$N^{-}PGPR^{-}$ (15 DAT)	1.5^{ab}	Na	na	na	na
$N^{-}PGPR^{-}$ (45DAT)	2.4^{d}	Na	na	na	na
$N^{-}PGPR^{-}$ (75DAT)	2.03^{c}	33.32^{a}	20.12^{a}	0.48^{c}	3.22^{a}
$N^{+}PGPR^{+}$ (15DAT)	1.4^{ab}	Na	na	na	na
$N^{+}PGPR^{+}$ (45 DAT)	3.23^{e}	Na	na	na	na
$N^{+}PGPR^{+}$ (75 DAT)	5.12^{g}	37.12^{c}	28.54^{c}	0.38^{a}	4.08^{c}
N^{+} $PGPR^{-}$ (15 DAT)	1.23^{a}	Na	na	na	na
N^{+} $PGPR^{-}$ (45 DAT)	2.33^{d}	Na	na	na	na
N^{+} $PGPR^{-}$ (75DAT)	4.54^{f}	36.16^{b}	26.15^{b}	0.42^{b}	3.98^{b}

Table 3.5
Effect of PGPR on the plant Chlorophyll content and Glutamine synthetase (GS) in response to different nitrogen treatment conditions. Mean values marked with different alphabets are significantly different at p<0.001, as suggested by one way analysis of variance

Treatment Group	Root GS (µmole $g^{-1}FW$)	Shoot GS (µmole $g^{-1}FW$)	Chlorophyll a (mg $g^{-1}FW$)	Chlorophyll b (mg $g^{-1}FW$)
$N^{-}PGPR^{+}$ (15 DAT)	13^{g}	1834^{k}	1.5^{d}	0.8^{c}
$N^{-}PGPR^{+}$ (45 DAT)	23^{h}	2122^{l}	1.9^{f}	0.9^{d}
$N^{-}PGPR^{+}$ (75 DAT)	17^{f}	121^{c}	2.3^{g}	1.13^{g}
N^{-} $PGPR^{-}$ (15 DAT)	8^{e}	712^{f}	1.1^{a}	0.5^{a}
$N^{-}PGPR^{-}$ (45DAT)	12^{bc}	549^{e}	1.1^{a}	0.7^{b}
N^{-} $PGPR^{-}$ (75DAT)	7^{a}	109^{b}	1.2^{abc}	0.7^{b}
$N^{+}PGPR^{+}$ (15DAT)	11^{cd}	1134^{h}	1.0^{a}	0.87^{c}
$N^{+}PGPR^{+}$ (45 DAT)	16^{cd}	1056^{g}	1.3^{c}	0.96^{de}
$N^{+}PGPR^{+}$ (75 DAT)	7^{a}	78^{a}	1.9^{f}	0.98^{de}
$N^{+}PGPR^{-}$ (15 DAT)	13^{h}	1544^{i}	1.6^{d}	0.81^{c}
$N^{+}PGPR^{-}$ (45 DAT)	15^{h}	1623^{j}	1.9^{f}	0.92^{d}
$N^{+}PGPR^{-}$ (75DAT)	10^{f}	212^{d}	2.0^{fg}	0.92^{d}

3.3.3.1. Chlorophyll content

In all the treatment regimes, Chlorophyll a and b contents were gradually increased from 15 to 75 of DAT. The level of chlorophyll a in $N^{-}PGPR^{+}$and $N^{+}PGPR^{-}$ plants was found to be comparable and was greater than that of $N^{+}PGPR^{+}$ plants at 45 DAT. However, at 75 DAT, the level of both chlorophyll a and b (2.3 and 1.13 mg g^{-1} FW, respectively) of $N^{-}PGPR^{+}$ plants were greater than those of $N^{+}PGPR^{+}$ plants (1.9 and 0.98 mg g^{-1} FW), whereas these pigments were present in almost equal quantity in plants with $N^{+}PGPR^{-}$ and $N^{+}PGPR^{+}$ treatments (Table 3.5)**.**

3.3.3.2. Determination of GS activity

The effect of application of various NFs on plant ammonia assimilation was determined by monitoring the GS activity of root and shoot tissue. From the results of Table-3.5, it is evident that the shoot exhibited significantly higher enzymatic activity than root. In general, GS activity was markedly higher in plants of all the treatment groups till 45 DAT, followed by a sharp decline during the later period of cultivation. At 45 DAT, roots and shoots of $N^{-}PGPR^{+}$ plants possessed highest GS activity followed by those of $N^{+}PGPR^{-}$ and $N^{+}PGPR^{+}$ plants. However, GS activity of the root and shoot of mustard plants under $N^{-}PGPR^{-}$ treatment was least and it was found to decline continuously during the cultivation time period.

3.3.4. Effect of application of RSM optimized NFs on plant growth and yield

The effect of three variables, N concentration (A), inoculum volume of strain1 i.e. *C. davisae* RS3 (B) and inoculum volume of strain 2 i.e. *K. pneumoniae* RS26 (C) of the NF, on plant growth (carbohydrate and protein content of shoot) and seed yield was investigated. Optimal levels of above variables were determined using CCRD based RSM. According to the CCRD, 20 experimental runs were conducted for the maturation stage (75 DAT) of mustard plant. The experimental design with actual and predicted values of response variables are presented in Table 3.6.

3.3.4.1 RSM optimization of carbohydrate content of shoot

Analysis of variance (ANOVA) was conducted for the experimental design used to optimize the carbohydrate content of shoot. The result in Table 3.6 shows the constructed model to be highly significant at the level of <0.000, having F ratio of 30.86, implying that the model could efficiently predict the response variables. Within the model, B (strain1), AB (N conc. x strain1), AC (N conc. x strain2), BC (strain1 x strain2), A^2 (N conc.^{2}) and B^2 (strain1^2) are the highly significant model terms. Using the actual result of the carbohydrate content of shoot, a second order polynomial regression equation was generated as mentioned below in coded (Eq. 7) and actual terms (Eq. 8).

Carbohydrate content of shoot (mg g^{-1} FW) = +8.28 - 0.67 x A + 2.31 x B + 0.62 x C-1.48 x A x B + 2.00 x A x C + 1.86 x B x C + 1.66 x A^2+ 0.79 x B^2- 0.64 xC^2……. (Eq.7)

Carbohydrate content of shoot (mg g^{-1} FW) = + 12.66 -2.45 x N conc. - 0.05x strain1-0.13 x strain2 -0.03 x N conc. x strain1 + 0.04 x N conc. x strain2 + 4.65E-003 x strain1 x strain2 + 0.32 x $Nconc.^2$ + 1.98E-003 x $strain1^2$-1.60E -003 x $strain2^2$…..(Eq. 8)

The fit of the model was evaluated with the help of various criteria, such as 'Lack of Fit' value, coefficient of determinant (R^2), adjusted R^2, predicted $R^2$and adequate precision (Table 3.7). The R^2 value of 0.9652 implies that the model could explain the 96% variability of the data. The predicted R^2 values are also in reasonable agreement with the adjusted R^2. Moreover, the high signal to noise ratio of 18.03 as explained by adequate precision, the insignificant 'Lack of Fit' and value of $p<0.06$ suggest that the model can be efficiently used to navigate the design space.

As can be seen from the result of Fig 3.7 that the data points for the observed carbohydrate content of shoot (response) and the model predicted carbohydrate content of shoot are split by 45°, indicating reasonable agreement of the actual response and the model predicted response.

Table 3.6.
Actual and predicted values with the experimental run. [A]N Concentration (mM), [B] Strain 1(% v/v), [C] Strain 2(% v/v), [X]Shoot Carbohydrate (mg/g FW), [Y]Shoot Protein (mg/g FW), [Z] Seed yield (g/plant)

Run	A	B	C	X_{actual}	$X_{predicted}$	Y_{actual}	$Y_{predicted}$	Z_{actual}	$Z_{predicted}$
1	2.75	30.00	30.00	8.64	8.82	716	723.62	4.50	4.60
2	0.50	50.00	10.00	14.92	14.08	420	414.47	5.37	5.37
3	2.75	30.00	30.00	8.64	8.82	716	723.62	4.50	4.60
4	2.75	30.00	30.00	7.63	8.82	770	723.62	4.50	4.60
5	2.75	30.00	30.00	7.60	8.82	710	723.62	5.39	4.65
6	0.50	50.00	50.00	16.00	15.04	824	819.25	8.10	8.20
7	5.00	10.00	10.00	7.36	7.83	560	551.35	2.65	2.65
8	6.53	30.00	30.00	12.00	11.85	612	635.51	4.30	4.38
9	2.75	30.00	30.00	8.64	8.82	717	723.62	4.50	4.60
10	5.00	50.00	50.00	15.48	14.74	523	498.95	7.00	6.90
11	0.00	30.00	30.00	13.28	14.12	670	665.44	6.50	6.40
12	5.00	50.00	10.00	6.00	5.77	470	460.17	4.10	4.09
13	0.50	10.00	50.00	4.00	3.74	430	426.43	6.60	6.64
14	2.75	30.00	30.00	8.64	8.82	716	723.62	4.50	4.60
15	2.75	30.00	63.64	6.80	7.52	471	488.46	7.70	7.60
16	2.75	30.00	0	5.44	5.42	320	321.49	3.08	3.05
17	0.50	10.00	10.00	9.96	10.21	256	266.65	3.90	3.98
18	2.75	63.64	30.00	13.00	14.41	513	532.80	6.50	6.54
19	2.75	0.00	30.00	7.36	6.64	280	279.15	4.17	4.09
20	5.00	10.00	50.00	9.00	9.36	353	345.13	5.40	5.30

Table 3.7
ANOVA Table for carbohydrate response.

Source	Sum of Squares	Df	Mean Squares	F value	p-value Prob>F	Remarks
Model	218.27	9	24.25	30.86	<0.0001	Significant
A	6.19	1	6.19	7.87	0.0186	
B	72.96	1	72.96	92.84	<0.0001	
C	5.32	1	5.32	6.78	0.0264	
AB	17.52	1	17.52	22.30	0.0008	
AC	32.00	1	32.00	40.72	<0.0001	
BC	27.68	1	27.68	35.22	0.0001	
A^2	39.91	1	39.91	50.78	<0.0001	
B^2	9.09	1	9.09	11.57	0.0068	
C^2	5.92	1	5.92	7.54	0.0206	
Residual	7.86	10	0.79			
Lack of fit	6.46	5	1.29	4.61	0.06	Not significant
Pure Error	1.40	5	0.28			
Cor total	226.13	19				
R^2 =0.9652, Adj-R^2= 0.9340, Pred-R^2=0.7739, A deq precision=18.032						

3.3.4.2. RSM optimization of protein content of shoot

The result in Table 3.7 shows the ANOVA conducted for the experimental design used to optimize the protein content of shoot. The constructed model was found to be highly significant at the level of <0.0001, having F ratio of 131.45, which implies that the model could efficiently predict the response variables. Within the model B (strain1), C (strain 2) AB (N conc. x strain1), AC (N conc. x strain2), BC (strain 1 x strain2), A^2 (N conc.^{2}), B^2 (strain1^2) and C^2 (strain2^2) are highly significant model terms. Using the actual result of the plant shoot protein content a second order polynomial regression equation was generated as shown in the equations (9) and (10) in coded and actual terms, respectively.

Protein content of shoot (mg g^{-1} FW) = + 723.62 - 0.89 x A + 75.41 x B + 49.64 x C - 59.75 x A x B + 91.50 x A x C + 61.25 x B x C - 25.86 x A^2 - 112.31 x B^2 - 112.66 xC^2……(Eq. 9)

Protein content of shoot (mg g^{-1} FW) = - 137.35 + 124.97 x N conc.+ 19.67 x strain1-20.37 x strain2- 1.32 x N conc. x strain1 - 2.03 x N conc. x strain2 + 0.15 x strain1 x strain2 -5.10 x N conc.^{2} - 0.28 x strain 1^2 - 0.28 x strain2^2..... (Eq. 10)

The fit of the model was evaluated on the basis of 'Lack of Fit' value, coefficient of determinant (R^2), adjusted R^2, predicted $R^2$and adequate precision (Table 3.8). The R^2 value of 0.9916 implies that the model could explain the 99% variability of the data. The predicted R^2 values are also in

reasonable agreement with the adjusted R^2. A high signal to noise ratio of 35.59 as explained by adequate precision and insignificant 'Lack of Fit' value of p=0.89 suggest that the model can be efficiently used to navigate the design space. The results in Fig 3.8 show that data points for the observed protein content (response) and the model predicted protein content are split by 45^o, indicating reasonable agreement between the actual response and the model predicted response.

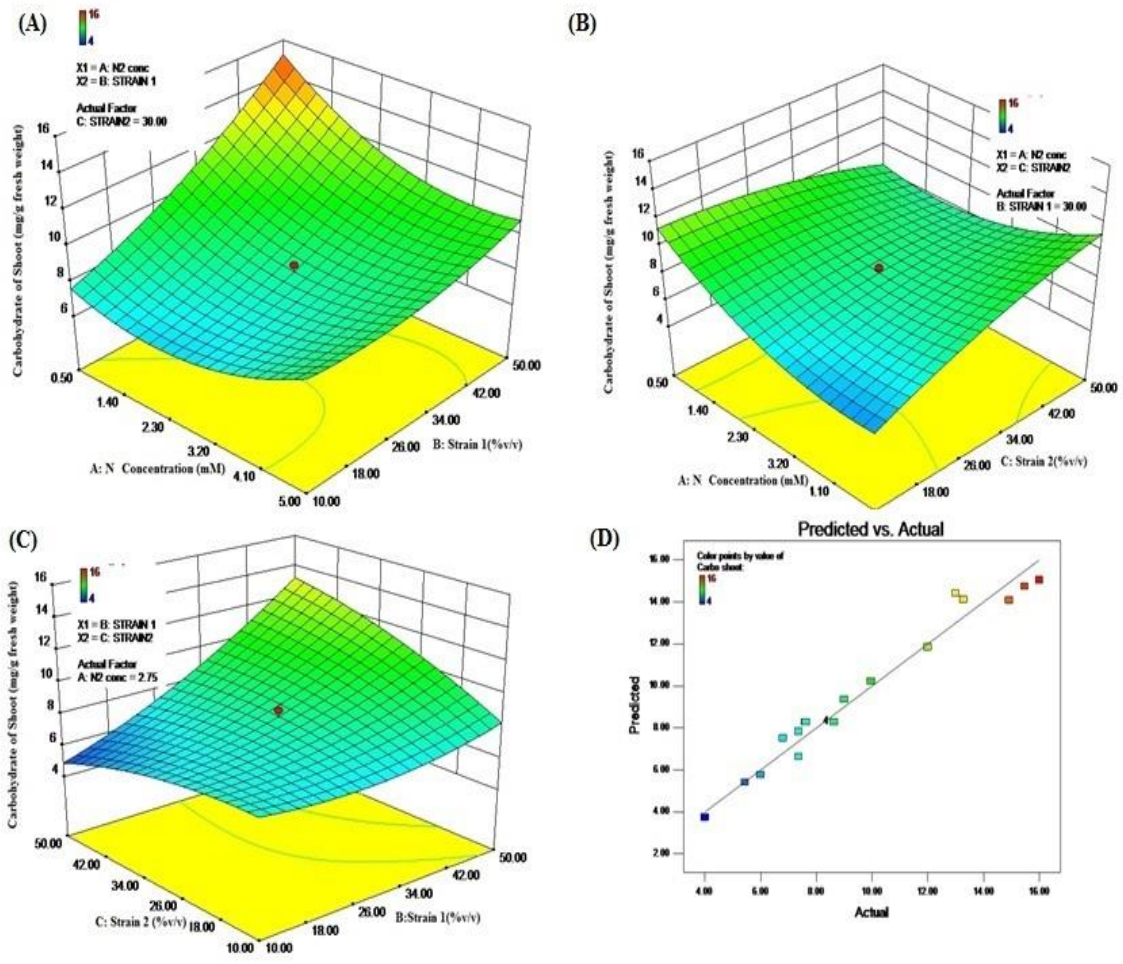


Fig 3.7: Response surface curves of carbohydrate content of shoot of Brassica campestris cv. B9, showing interaction between [A] N concentration (mM) and strain 1(RS3) inoculum volume (% v/v), [B] N concentration (mM) and strain 2 (RS26) inoculum volume (% v/v), [C] strain 2 (RS26) inoculum volume (% v/v) and strain 1 (RS3) inoculum volume (% v/v), [D] model predicted versus actual carbohydrate content of shoot plot.

Table 3.8
ANOVA Table for protein response

Source	Sum of Squares	Df	Mean Squares	F value	p-value Prob>F	Remarks
Model	5.701E+005	9	63347.93	131.45	<0.0001	Significant
A	1081.72	1	1081.72	2.24	0.1650	
B	77661.15	1	77661.15	161.15	<0.0001	
C	33654.69	1	33654.69	69.83	<0.0001	
AB	28560.50	1	28560.50	59.26	<0.0001	
AC	66978.00	1	66978.00	138.98	<0.0001	
BC	30012.50	1	30012.50	62.28	<0.0001	
A2	9638.59	1	9638.59	20.00	0.0012	
B2	1.818E+005	1	1.818E+005	377.16	<0.0001	
C2	1.829E+005	1	1.829E+005	379.54	<0.0001	
Residual	4819.18	10	481.92			
Lack of fit	2266.35	5	453.27	0.89	0.5504	Not significant
Pure Error	2552.83	5	510.57			
Cor total	5.750E+005	19				
R^2 = 0.9916, Adj-R^2= 0.9841, Pred-R^2=0.9634, A deq precision=35.60						

3.3.4.3. RSM optimization of seed yield per plant

ANOVA was conducted for the experimental design used to optimize the seed yield. The results presented in the Table 3.8 show the constructed model to be highly significant at a level of <0.0001, having F ratio of 66.20, which implies that the model could efficiently predict the response variables. Within the model, A (N conc.), B (strain1), C (strain 2), A^2 (N conc.^{2}), B^2 (strain1^2) and C^2 (strain2^2) are the highly significant model terms. Using the actual result regarding the seed yield of mustard plant, a second order polynomial regression equation was generated as presented in Eq. (11) and (12) in coded and actual term, respectively.

Seed yield plant^{-1}= +4.65 - 0.62 x A + 0.73 x B + 1.38 x C + 0.0010 x A x B + 0.028 x A x C + 0.023 x B x C + 0.26 x A^2 + 0.24 x B^2 + 0.26 x C^2…….....(Eq. 11)

Seed yield plant^{-1}= +3.86560 – 0.58432 x N conc. - 1.40736E - 003 x strain1+0.027232 x strain2 + 2.22222 E-004 x N conc. x strain1 + 6.11111E-004 x N conc. x strain2 + 5.62500E-005 x strain1 x strain2 + 0.051283 x N conc.^{2} + 5.91592E-004 x strain1^2+6.40206E-004 x strain2^2……..(Eq. 12)

The fit of the model was evaluated on the basis of criteria, like 'Lack of Fit' value, coefficient of determinant (R^2), adjusted R^2, predicted $R^2$and adequate precision (Table 3.9). The R^2 value of 0.9835 implies that the model could explain the 98% variability of the data. The predicted R^2 values are also in reasonable agreement with the adjusted R^2. A high signal to noise ratio of 29.50 as explained by adequate precision and the insignificant 'Lack of Fit' value of p=0.99

suggest that the model can be efficiently used to navigate the design space. The data points for observed seed yield (response) per plant and the model predicted seed yield per plant are split by 45^{o}, indicating the reasonable agreement of the actual response with the model predicted response (Fig 3.9).

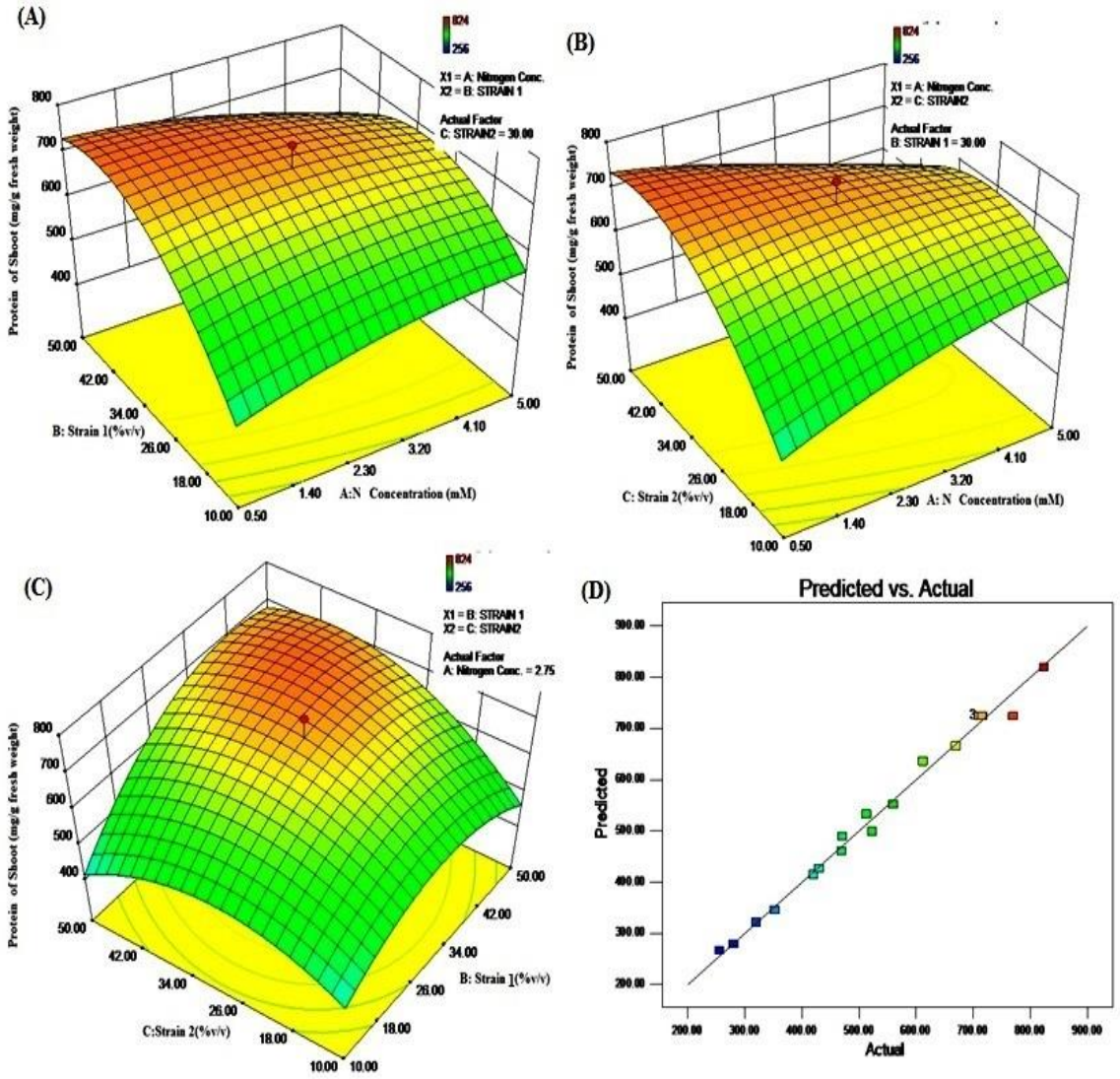


Fig 3.8: Response surface curves of protein content of shoot of Brassica campestris cv. B9., showing interaction between [A] N concentration(mM) and strain 1(RS3) inoculum volume (% v/v), [B] N concentration (mM) and strain 2 (RS26) inoculum volume (% v/v), [C] strain 2(RS26) inoculum volume (% v/v) and strain 1(RS3) inoculam volume (% v/v), [D] model predicted versus actual protein content of shoot plot.

Table 3.9
ANOVA Table for seed yield response.

Source	Sum of Squares	Df	Mean Squares	F value	p-value Prob>F	Remarks
Model	40.85	9	4.54	66.20	<0.0001	Significant
A	5.32	1	5.32	77.52	<0.0001	
B	7.23	1	7.23	105.48	<0.0001	
C	26.02	1	26.02	379.44	<0.0001	
AB	8.000E-004	1	8.000E-004	0.012	0.9161	
AC	6.050E-003	1	6.050E-003	0.088	0.7725	
BC	4.050E-003	1	4.050E-003	0.059	0.8129	
A2	0.97	1	0.97	14.17	0.0037	
B2	0.81	1	0.81	11.77	0.0064	
C2	0.95	1	0.95	13.78	0.0040	
Residual	0.69	10	0.069			
Lack of fit	0.026	5	5.118E-003	0.039	0.9986	Not significant
Pure Error	0.66	5	0.13			
Cor total	41.54	19				
R^2 =0.9835, Adj-R^2=0.9686, Pred- R^2= 0.9724, Adeq precision= 29.508						

3.3.4.4. Interpretation of interaction effect between independent variables on plant growth and yield

The response surface plots and their contour plots were generated from the second order polynomial regression equations in order to investigate the effect of interactions among variables on optimal level of response variables.

The response surface plot A vs. B (Fig 3.7) for carbohydrate content as response variables indicated a significant interaction between the N conc. (A) and strain1 inoculum volume (B), which was further justified by ANOVA with P value of 0.0008. The highest shoot carbohydrate content was observed when the inoculum volume of strain1 was increased to 50 % (v/v) and the N conc. was reduced to 0.5 mM. The carbohydrate content did not change significantly, when the inoculum volume of strain1 was varied between 10-30 % v/v, keeping the N conc. fixed at 0.5 mM. The results in Fig.3.8 represent the effect of interaction between A and B on the shoot protein content as response variable. The protein content was maximum (713 mg mL^{-1}) with the treatment formulation containing 50 % (v/v) of strain1and 0.5 mM N. The plot also depicts that keeping the inoculum volume of strain1 fixed at 50% (v/v) and varying the N conc. between 0.50 to 5.0 mM resulted in a significant reduction of protein from 713 to 400 mg g^{-1}. Response surface plot of A vs. B for optimizing the seed yield (g $plant^{-1}$) response was constructed (Fig.3.9). The plot showed non-significant (P=0.91) interaction between the two variables, however, individually the two variables contributed significantly to the seed yield. When the N

conc. was reduced from 5 mM to 0.5 mM, the seed yield enhanced significantly (P<0.0001) and similarly, when inoculum volume of strain1 in the formulation was increased from 10 to 50% (v/v), seed yield plant^{-1} was also enhanced

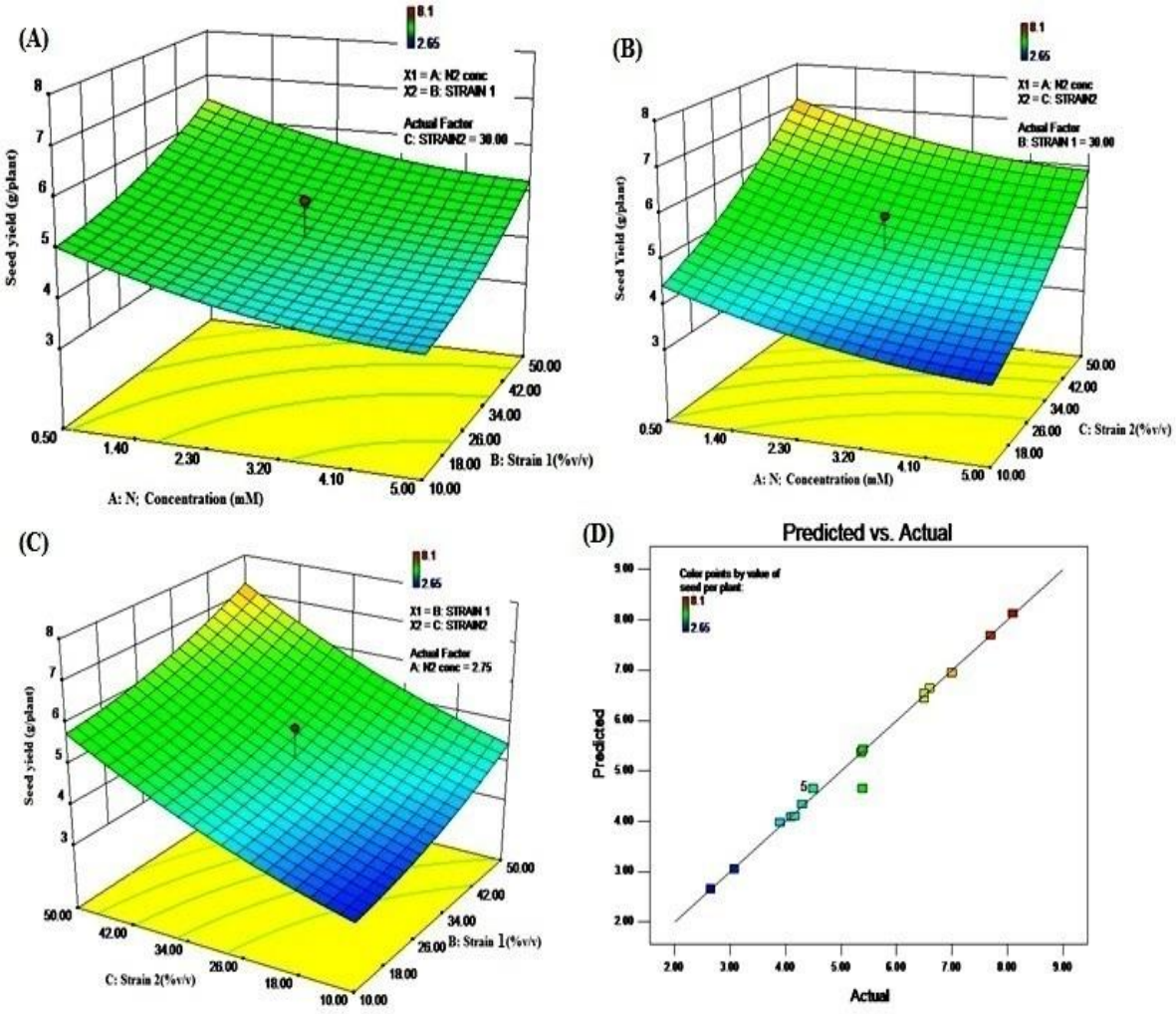


Fig 3.9: Response surface curves of seed yield of Brassica campestris cv. B9., showing interaction between [A] N concentration (mM) and strain 1(RS3) inoculum volume (% v/v), [B] N concentration (mM) and strain 2(RS26) inoculum volume (% v/v), [C] strain 2 (RS26) inoculum volume (% v/v) and strain 1(RS3) inoculum volume (% v/v), [D] model predicted versus actual seed yield plot

The influence of interaction among N conc. (A) and inoculum volume of strain 2 (C) on the response variables, shoot carbohydrate and protein content, and seed yield were also evaluated. From the graphs of A vs. C (Fig 3.7B, 3.8B, 3.9 B), it can be seen that the interaction effect between the two variables is highly significant (P<0.0001). A significant increment in shoot carbohydrate and protein contents, and seed yield was obtained at 50% (v/v) of strain 2 and 0.5 mM N. Response

surface plots between B vs. C for carbohydrate, protein and seed yield responses showed that the treatment of plants with RS3 (B) and RS26 (C) at 50% (v/v) each supported maximum carbohydrate and protein contents, and seed yield respectively (Fig 3.7 C, 3.8 C, 3.9 C). Although the interaction effect between the variables B and C was not significant (P=0.81), individually these two variables showed a significant effect on the seed yield.

Table 3.10
Validity of the model with Actual and predicted values of the experimental run
[A]N Concentration (mM), [B] Strain 1(% v/v), [C] Strain 2(% v/v), [X] Carbohydrate of Shoot (mg g^{-1} FW), [Y] Protein content of Shoot (mg g^{-1} FW), [Z] Seed yield (g $plant^{-1}$)

Run	A	B	C	X_{actual}	$X_{predicted}$	Y_{actual}	$Y_{predicted}$	Z_{actual}	$Z_{predicted}$
1	0.50	50	37.49	14.79	15.29	800	817.204	7.65	7.98
2	5.00	50	50	13.76	14.73	550.43	538.21	7.80	8.01
3	0.73	50	37.30	13.89	14.81	811.76	794.88	6.98	7.54
4	0.50	50	10.14	14.72	14.08	767.87	737.67	4.98	5.76
5	**0.50**	**50.00**	**50.00**	**16.00**	**15.04**	**824**	**819.25**	**8.10**	**8.20**

3.3.4.5. The validity of the model and prediction of optimized variables

The validity of the model was tested by carrying out 5 different experimental runs using model optimized independent variables, in order to maximize the shoot carbohydrate and protein content, and seed yield of the mustard plant. The results (Table 3.10) clearly show that the actual values are very close to the predicted values and thus the model was successfully validated. The experimental runs suggested that the application of NF containing N at 0.5 mM and strain 1 and strain 2 at 50% (v/v) to the cultivation of mustard plant, yielded actual response of shoot carbohydrate content (16 mg g^{-1}FW), shoot protein content (824 mg g^{-1}FW) and seed yield (8.10 g $plant^{-1}$), which are quite close to the model predicted response comprising shoot carbohydrate content of shoot (15.04 mg g^{-1} FW), shoot protein content (819.25 mg g^{-1}FW), seed yield (8.20 g $plant^{-1}$). Hence, the CCRD based RSM models were accurate and reliable in predicting the growth and seed yield of *Brassica* plant.

3.3.4.6. Comparison of RSM treatment approach with General treatment approach

A comparison of the effect of general and RSM based approaches of plant NF treatments to the growth and yield of mustard plant indicated that carbohydrate (16 mg g^{-1} FW) and protein (824 mg g^{-1} FW) contents and seed yield (8.10 g $plant^{-1}$), GS activity (211 µmole g^{-1} FW) and Chlorophyll a (2.7 mg g^{-1} FW) of RSM based treatment plants were significantly greater than that of the plant treated with general approach with carbohydrate content, protein contents, seed yield, GS activity and Chlorophyll a of 12.17 mg g^{-1} FW, 675.89 mg g^{-1} FW and 5.76 g $plant^{-1}$,121 µmole g^{-1} FW and 2.3 mg g^{-1} FW, respectively (Table 3.11, 3.12, 3.13, 3.14 and Fig 3.10).

Table 3.11

Comparison of effect of morphological parameters of the plants treated under RSM approach and $N^{-}PGPR^{+}$. Mean values marked with different alphabets are significantly different at $p<0.001$, as suggested by one way analysis of variance

Treatment Groups	Root FW (g)	Shoot FW (g)	Root length (cm)	Stem length (cm)
RSM (15 DAT)	0.54^{c}	0.86^{c}	2.16^{c}	10.5^{f}
RSM (45 DAT)	1.12^{g}	1.96^{i}	5.21^{g}	18.6^{j}
RSM (75 DAT)	1.24^{h}	2.05^{j}	8.78^{i}	25.45^{k}
$N^{-}PGPR^{+}$ (15 DAT)	0.43^{b}	0.76^{bc}	1.89^{b}	8.56^{d}
$N^{-}PGPR^{+}$(45 DAT)	1.08^{f}	1.43^{g}	4.36^{e}	14.87^{i}
$N^{-}PGPR^{+}$(75 DAT)	1.17^{g}	1.56^{h}	6.76^{h}	20.12^{jk}

Table 3.12

Effect of PGPR on the plant carbohydrate and protein content in response to different nitrogen treatment conditions. Mean values marked with different alphabets in each column are significantly different at $p<0.001$, as suggested by one way analysis of variance

Treatment Groups	Root Carbohydrate (mg g^{-1} FW)	Shoot Carbohydrate (mg g^{-1} FW)	Root Protein (mg g^{-1} FW)	Shoot Protein (mg g^{-1} FW)
RSM (15 DAT)	3.04^{d}	8.65^{e}	125.12^{d}	345.22^{f}
RSM (45 DAT)	5.98^{f}	9.08^{f}	432.43^{g}	621.22^{i}
RSM (75 DAT)	8.67^{h}	16.12^{i}	543.18^{h}	924.76^{j}
$N^{-}PGPR^{+}$ (15 DAT)	2.12^{c}	6.34^{c}	96.17^{c}	235.32^{e}
$N^{-}PGPR^{+}$ (45 DAT)	4.34^{e}	8.67^{e}	234.37^{e}	543.72^{h}
$N^{-}PGPR^{+}$ (75 DAT)	6.76^{g}	12.17^{h}	378.67^{f}	675.89^{i}

Table 3.13
Effect of PGPR on the plant seed yield in response to different treatment conditions
Mean values marked with different alphabets in each column are significantly different at p<0.001, as suggested by one way analysis of variance. 'na'= not available

Treatment Groups	No. of branches	No.of Siliqua/plant	Seed/siliqua	100 seed weight (g)	Seed yield/plant (g)
RSM (15 DAT)	1.34^{ab}	Na	na	na	na
RSM (45 DAT)	3.12^{e}	Na	na	na	na
RSM (75 DAT)	5.03^{g}	42.13^{e}	37.6^{e}	0.51^{d}	8.10^{e}
$N^{-}PGPR^{+}$ (15 DAT)	1.21^{a}	Na	na	na	na
$N^{-}PGPR^{+}$ (45 DAT)	2.65^{d}	Na	na	na	na
$N^{-}PGPR^{+}$ (75 DAT)	4.23^{f}	39.40^{d}	30.12^{d}	0.48^{c}	5.76^{d}

Table 3.14
Comparison of Chlorophyll content and Glutamine synthetase (GS) of plants under RSM approach and best of general approach. Mean values marked with different alphabets are significantly different at p<0.001, as suggested by one way analysis of variance.

Treatment Groups	Root GS (µmole g^{-1} FW)	Shoot GS (µmole g^{-1}FW)	Chlorophyll a (mg g^{-1}FW)	Chlorophyll b (mg g^{-1} FW)
RSM (15 DAT)	15^{cd}	2188^{gh}	1.7^{de}	0.99^{de}
RSM (45 DAT)	29^{cd}	227^{8h}	2.2^{g}	1.03^{f}
RSM (75 DAT)	19^{bc}	211^{c}	2.7^{h}	1.32^{i}
$N^{-}PGPR^{+}$ (15 DAT)	13^{g}	1834^{k}	1.5^{d}	0.8^{c}
$N^{-}PGPR^{+}$ (45 DAT)	23^{h}	2122^{l}	1.9^{f}	0.9^{d}
$N^{-}PGPR^{+}$ (75 DAT)	17^{f}	121^{c}	2.3^{g}	1.13^{g}

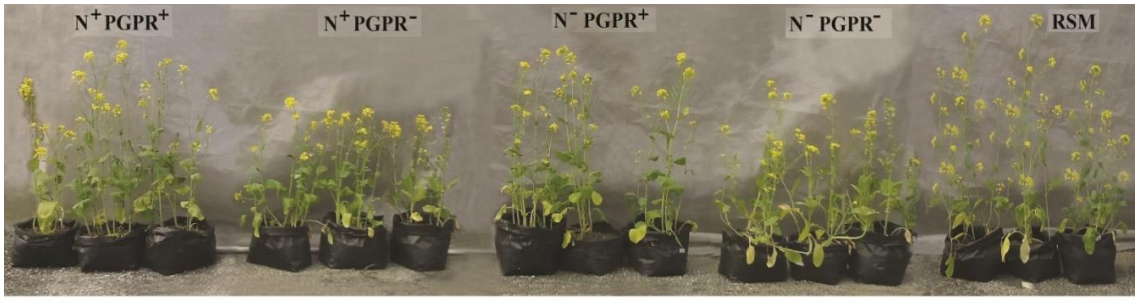


Fig 3.10. Plants treated with PGPR supplementation under differential N inputs

3.3.5. Differential transcriptomics analysis of mustard plants under NF with highest yield

Mustard plants treated with NFs showing highest yield was subjected to transcriptomic analysis in order to gain an insight into the role of NF in plant growth promotion. For this, total RNA was isolated from roots of highest yield plants treated with RSM optimized NF containing 0.5 mM $(NH_4)_2SO_4$ and and 50% v/v of each strains RS3 and RS26 and the plants treated with NF containing only 50 mM $(NH_4)_2SO_4$ inorganic i.e (N^+PGPR^-) served as control. For transcriptomics analysis, cDNA library constructed from mRNA purified from the total RNA was sequenced using Illumina-based 2 × 75bp paired-end reads sequencing (Fig 3.11). Henceforth, the plants treated with RSM optimized NF and those with only inorganic N containing NF will be designated as 'treated' and 'control', respectively. In total of 25,146,303 reads and 26,268,092 reads were produced for the libraries of control and treated plants, generating 7.73 Gb of data. The sequences were deposited in the National Center for Biotechnology Information (NCBI) which can be accessed from the sequence read archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under the accession no. PRJNA565414.

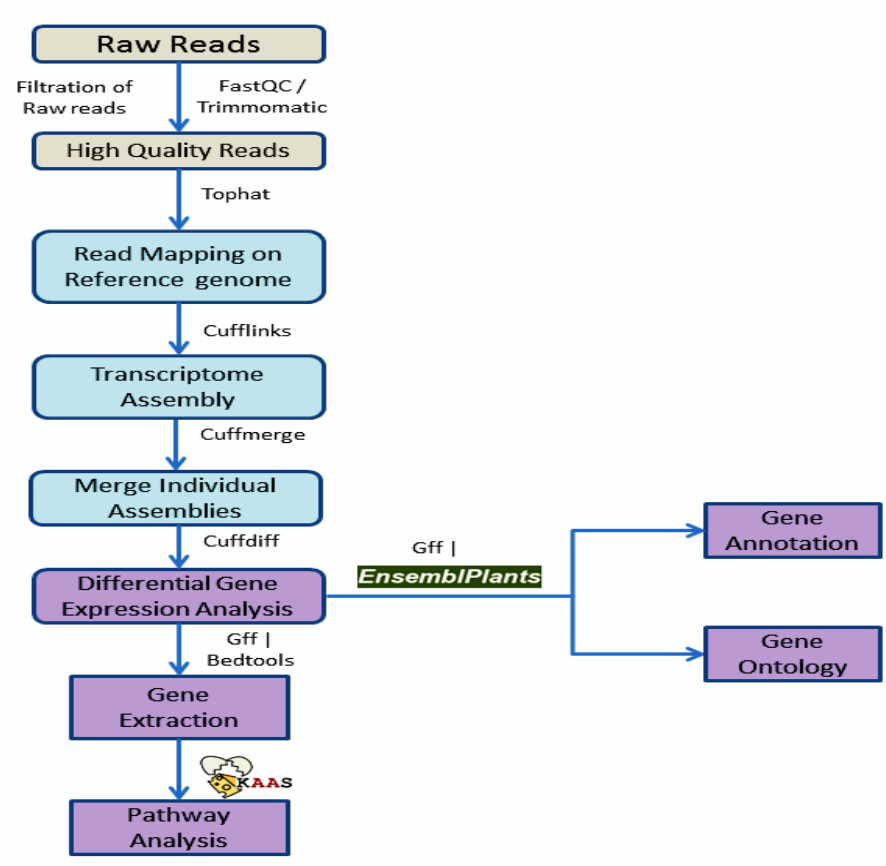


Fig. 3.11. Flow diagram of Illumina based transcriptomics

The high quality reads of the two samples were mapped on the reference genome of *Brassica rapa*, using TopHat v2.1.1 with default parameters. The mapping statistics was found to be 43.5% in case of control group of plant whereas 42.5% mapping statistics was noted for PGPR treated plant group. The results of differential gene expression (DGE) analysis showed that 25,088 protein coding genes were expressed in both control and NF treated group of plants, whereas 357 genes were exclusively expressed in NF treated group, 351 genes were exclusively expressed in control group of plants. The expression levels of total 556 genes were upregulated and 690 genes were downregulated in the NF treated plants as compared to the control group (Fig 3.12).

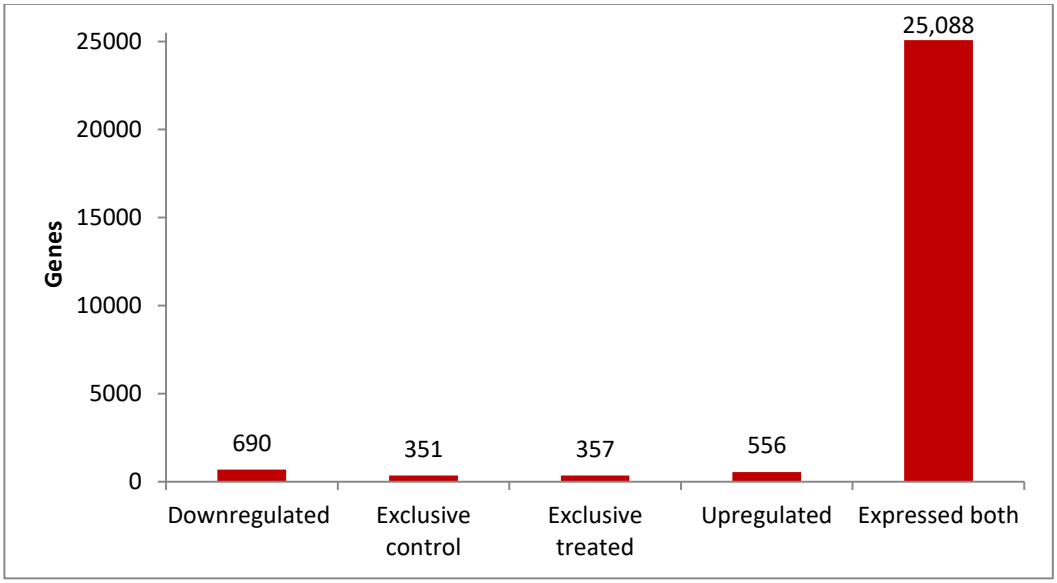


Fig.3.12. Differential gene expression analysis of protein coding genes of control and PGPR treated group of plant

An average linkage hierarchical cluster analysis was performed on 45 differentially expressed genes for the combination of control and treated groups, which has been shown as the heat map in the Figure 3.13. In the heat map each horizontal line refers to a gene and the color represent the logarithmic intensity of the gene expression. It can be seen that the application of RSM optimized NF to the plant resulted in upregulation of several genes encoding proteins or enzymes to be directly or indirectly associated with plant productivity like Lys/His Transporter 7, ammonia transporter, NADP-dependent oxidoreductase, chorismate mutase 1, WRKY35 transcription factor, mitochondrial phosphate transporter, amino acid transporter family protein, CTP synthase, phenylalanine ammonia-lyase, inorganic phosphate transmembrane transporter, flowering promoting factor 1, growth regulating factor 2, nitrate reductase 1, glutathione peroxidase, transmembrane transporter and $NADP^+$ isocitrate dehydrogenase are responsible for

plant growth and yield directly. In addition, several gene products with indirect effect on plant growth promotion by giving resistance against pathogens were also upregulated, such as UDP-glucosyl transferase family protein, glutathione S-transferase F3, mildew resistance locus O12, INH3, monooxygenase, seed storage/lipid Transfer Protein (LTP) family protein, disease resistance response and pleiotropic drug resistance 7. Those genes whose downregulation have significant role in plant growth and yield found in this study were cell wall / vacuolar inhibitor of fructosidase 1, WRKY18 trancription factor, pyruvate decarboxylase, senescence-associated protein-related and glutamine dumper 1. The amount of Fragments Per Kilobase of transcript per Million (FPKM) in both control and treated, log 2 fold change and the role of the genes are depicted in Table 3.18.

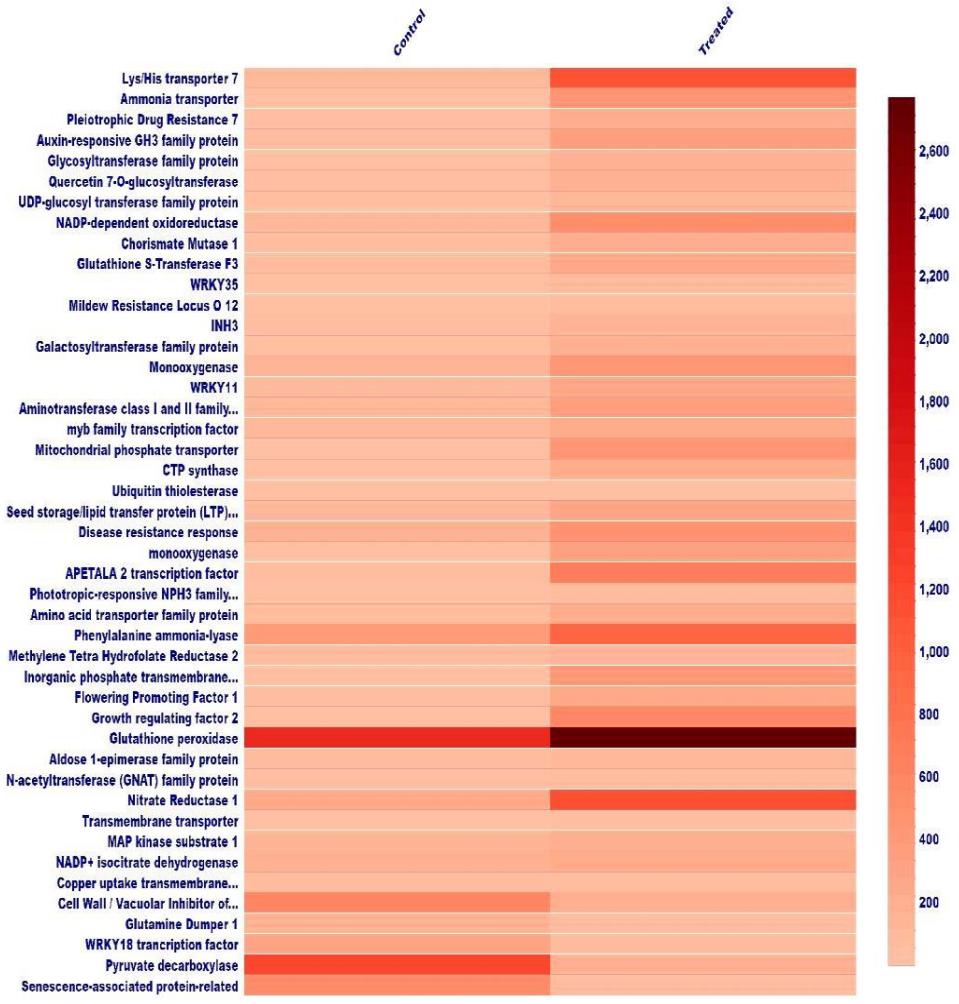


Fig. 3.13. Heat map of differentially expressed genes of control vs treated group

The result in Figure 3.14 depicts the differential expression of genes between control and treated plants in the form of scatter plot. In scatter plot each dot represents a gene. The vertical position

of each gene represents its expression level in the control samples while the horizontal position represents its expression level in the treated samples. The genes that fall above the diagonal are over-expressed and genes that fall below the diagonal are under expressed as compared to their median expression level in experimental grouping of the experiment.

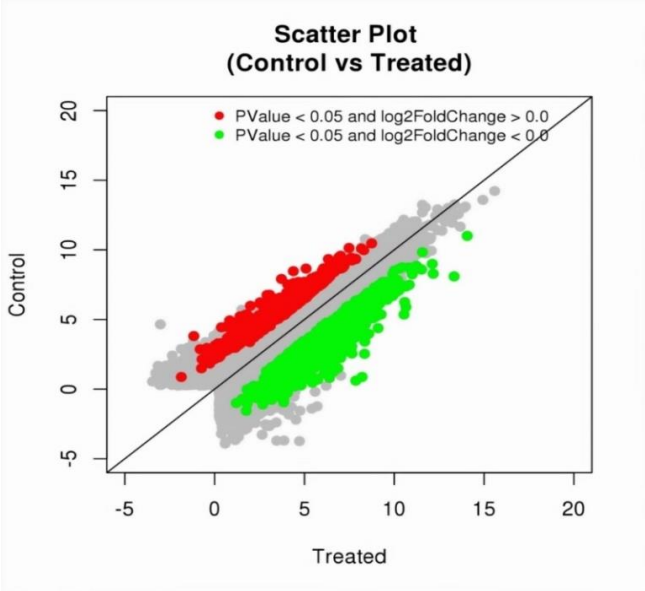


Fig. 3.14. Scatter plots of differentially expressed genes, green dots represent the down regulated and red dots represent upregulated genes in treated samples with respect to control group

3.3.5.1. Gene ontology (GO) analysis

GO annotation for the up-regulated, down-regulated, expressed in both and exclusively expressed genes for the control and treated samples were obtained from the Ensembl Plants database for *Brassica rapa*. The results in Table 3.15 give the information about the number of genes assigned to various GO domains, like biological process (BP), cellular component (CC) and molecular function (MF). The bar plots, depicting the GO distribution, were obtained through WEGO portal (http://wego.genomics.org.cn/cgi-bin/wego/inaex.pl).

Table 3.15
Statistics of Gene Ontology (GO) analysis

Description	Biological process (BP)	Cellular component (CP)	Molecular Function (MF)
Exclusive control	177	37	119
Exclusive treated	153	33	104
Expressed in both	27,282	7692	22,252
Upregulated in treated plant	632	96	598
Downregulated in treated plant	889	155	592

The genes that were downregulated and upregulated in the treated group with respect to control group are shown in the Fig 3.15 and Fig 3.16 respectively. Among the cellular components and biological processes, the expression of genes related to organelle, extracellular region part, localization, cell proliferation, detoxification, carbon utilization was increased, whereas genes encoding proteins for nucleiod and rhythmic processes were found to be downregulated. (Table 3.16).

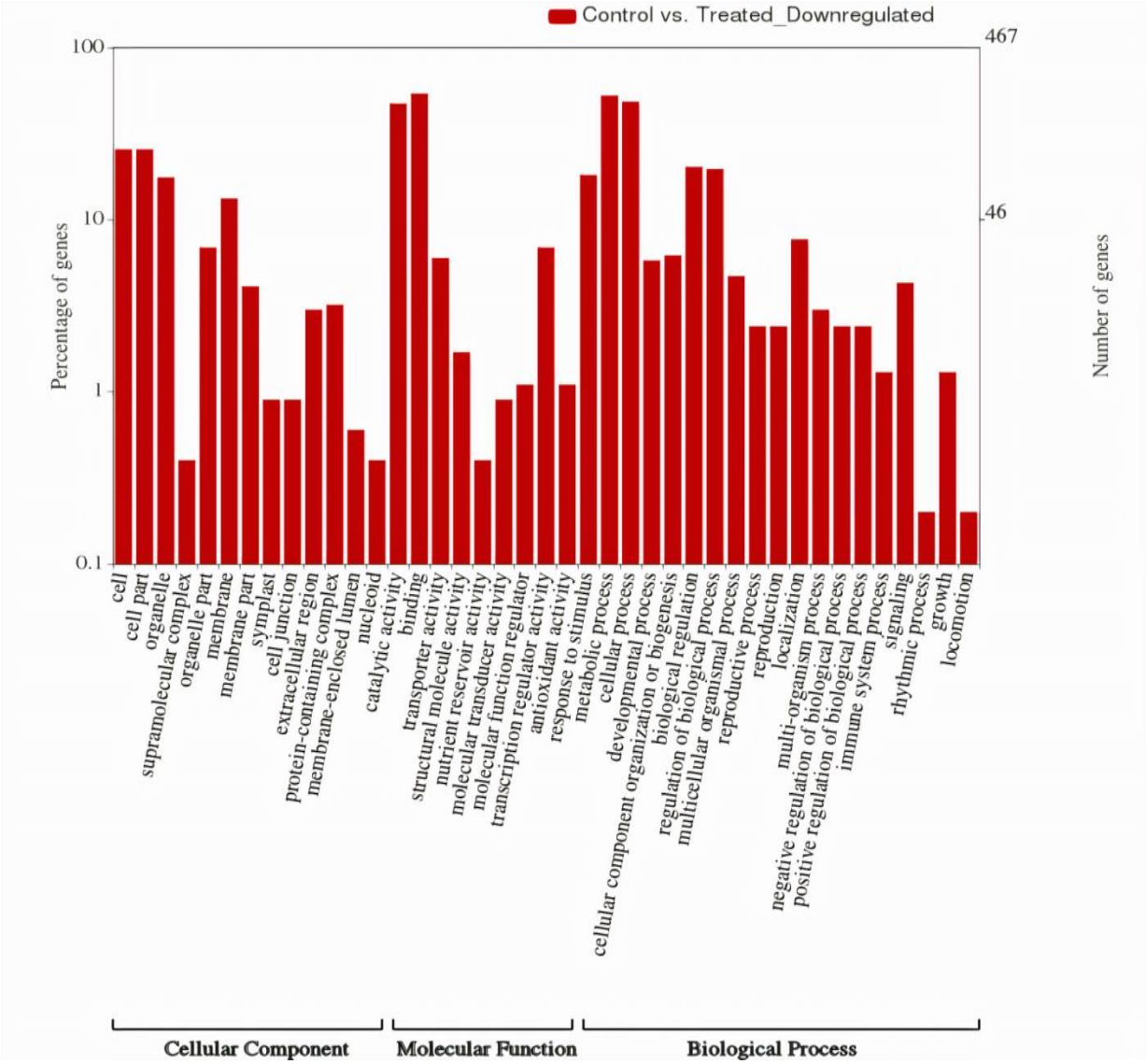


Fig. 3.15. WEGO plot showing the downregulated genes in PGPR treated plants as compared to control group.

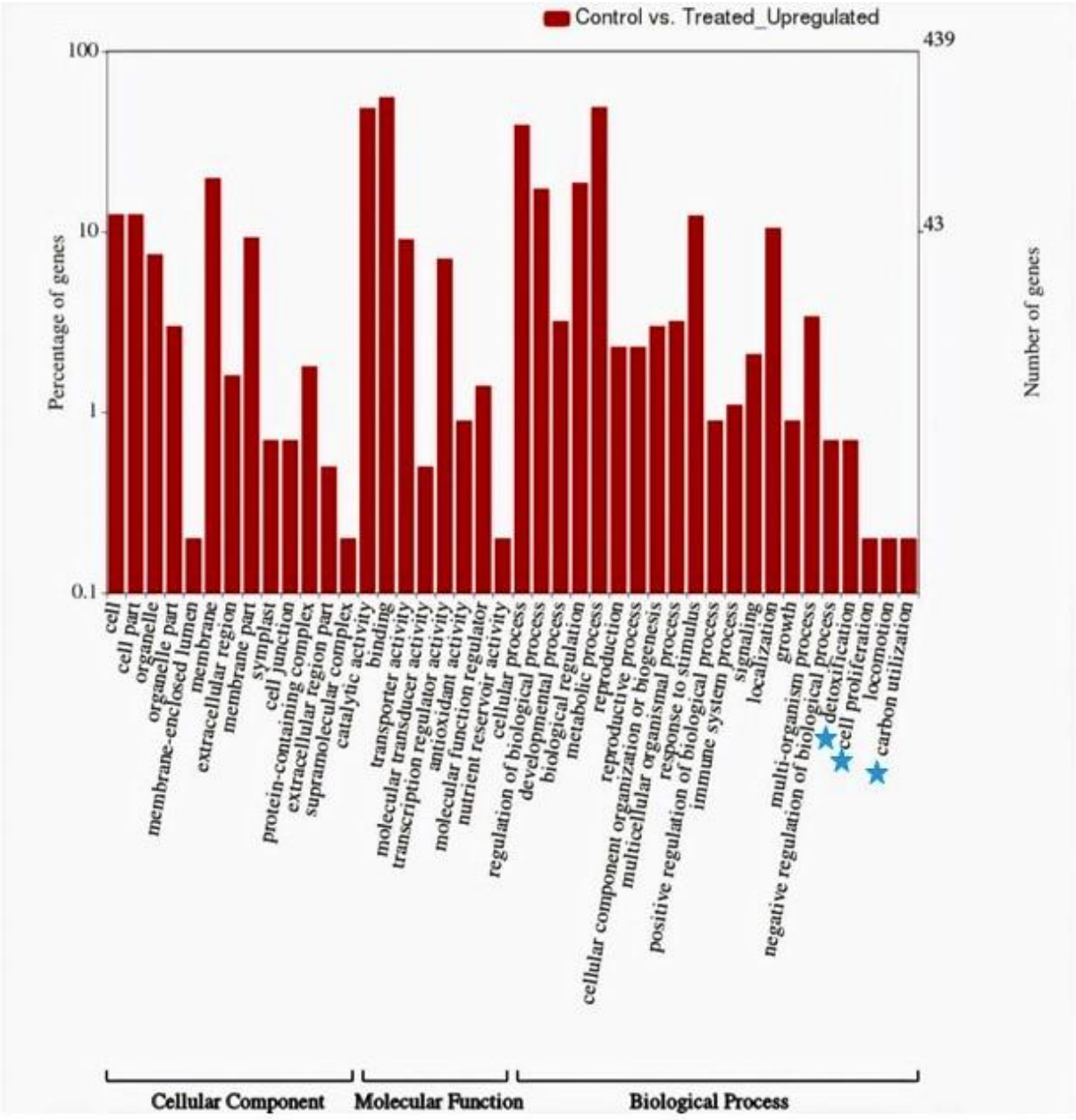


Fig 3.16. WEGO plot showing the upregulated genes in PGPR treated plants as compared to control group.

Table 3.16.
Comparison of the genes present in the three GO domains (cellular component molecular function and biological process)

Genes		Down Regulated in Treated	Up Regulated in Treated	Expressed in both	Exclusively Control	Exclusively treated
Cellular component	Cell	+	+	+	+	+
	Cell part	+	+	+	+	+
	Supramolecular complex	+	+	+	NA	NA
	Menbrane part	+	+	+	+	+
	Membrane	+	+	+	+	+
	Organelle	NA	+	+	+	+
	Organelle part	+	+	+	+	+
	Extracellular region	+	+	+	+	+
	Protein containing complex	+	+	+	+	+
	Extracellular region part	NA	+	+	NA	NA
	Symplast	+	+	+	+	+
	Cell junction	+	+	+	+	+
	Membrane enclosed lumen	+	+	+	+	+
	Nucleiod	+	NA	+	NA	NA
	Catalytic activity	+	+	+	+	+
Molecular function	Binding	+	+	+	+	+
	Structural molecule activity	+		+	+	+
	Transporter activity	+	+	+	+	+
	Nutrient reservoir activity	+	+	+	NA	NA
	Molecular transducer activity	+	+	+	NA	NA
	Molecular function regulator	+	+	+	+	+
	Transcription regulator activity	+	+	+	+	+
	Tranlation regulator activity	NA	NA	+	NA	NA
	Antioxidant activity	+	+	+	NA	+
	Molecular carrier activity	NA	NA	+	NA	NA
Biological processes	Developmental process	+	+	+	+	+
	Biological regulation	+	+	+	+	+
	Regulation of Biological process	+	+	+	+	+
	Multicellular organismal process	+	+	+	+	+
	Response to stimuli	+	+	+	+	+
	Localization	NA	+	+	+	+
	Signaling	+	+	+	+	+
	Cellular process	+	+	+	+	+
	Metabolic process	+	+	+	+	+
	Multi-organism process	+	+	+	+	+
	Immune system process	+	+	+	+	+
	Negative regulation of biological process	+	+	+	+	+
	Cellular component organization or biogenesis	+	+	+	+	+
	Reproductive process	+	+	+	+	+
	Reproduction	+	+	+	+	+
	Positive regulation of biological process	+	+	+	+	+
	Growth	+	+	+	+	+
	Cell proliferation	NA	+	+	NA	+
	Rhythmic process	+	NA	+	NA	+
	Locomotion	+	+	+	NA	NA
	Detoxification	NA	+	+	NA	NA
	Carbon utilization	NA	+	+	NA	NA
	Nitrogen utilization	NA	NA	+	NA	NA
	Pigmentation	NA	NA	+	NA	NA

'+'= present NA=Not Available

Table 3.17. **KEGG pathway classification**	
Pathways	**Gene counts**
Metabolism	
Carbohydrate metabolism	714
Energy metabolism	426
Lipid metabolism	402
Nucleotide metabolism	146
Amino acid metabolism	465
Metabolism of other amino acids	219
Glycan biosynthesis and metabolism	149
Metabolism of cofactors and vitamins	274
Metabolism of terpenoids and polyketides	141
Biosynthesis of other secondary metabolites	260
Xenobiotics biodegradation and metabolism	116
Genetic Information Processing	
Transcription	313
Translation	863
Folding, sorting and degradation	630
Replication and Repair	175
Environmental Information Processing	
Membrane Transport	41
Signal transduction	997
Signaling molecules and interaction	1
Cellular Processes	
Transport and catabolism	577
Cell growth and death	391
Cellular community- eukaryotes	98
Cellular community-Prokarotes	69
Cell motility	55
Organismal System	
Environmental adaptation	420

3.3.5.2. Metabolic pathway analysis

The functional annotations of the genes were carried out against the curated KEGG gene data base using KAAS (KEGG automatic annotation server). The KEGG ontology (KO) database of plants was used as the reference for pathway mapping. The result contains KO assignments and automatically generated KEGG pathways using KAAS BBH (bidirectional best hit) method using available database. In KASS, 25,834 genes were processed. These genes were classified into 24 functional pathway categories annotating 7,942 genes in KEGG. The full KEGG pathway classification are represented in Table 3.17.

The genes expressed exclusively in treated group were further analyzed for their affiliation to various metabolic pathways. Among these five genes were found to be associated with the phytohormone signal transduction pathways. They encoded Sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK2) ($p<0.05$), basic-leucine zipper (bZIP) transcription factor family protein ($p<0.05$), Auxin-responsive GH3 family protein (GH3) ($p<0.018$), ABA-responsive element binding factor (ABF) ($p<0.03$) and SAUR-like auxin-responsive protein family ($p<0.04$) (Fig. 3.17).

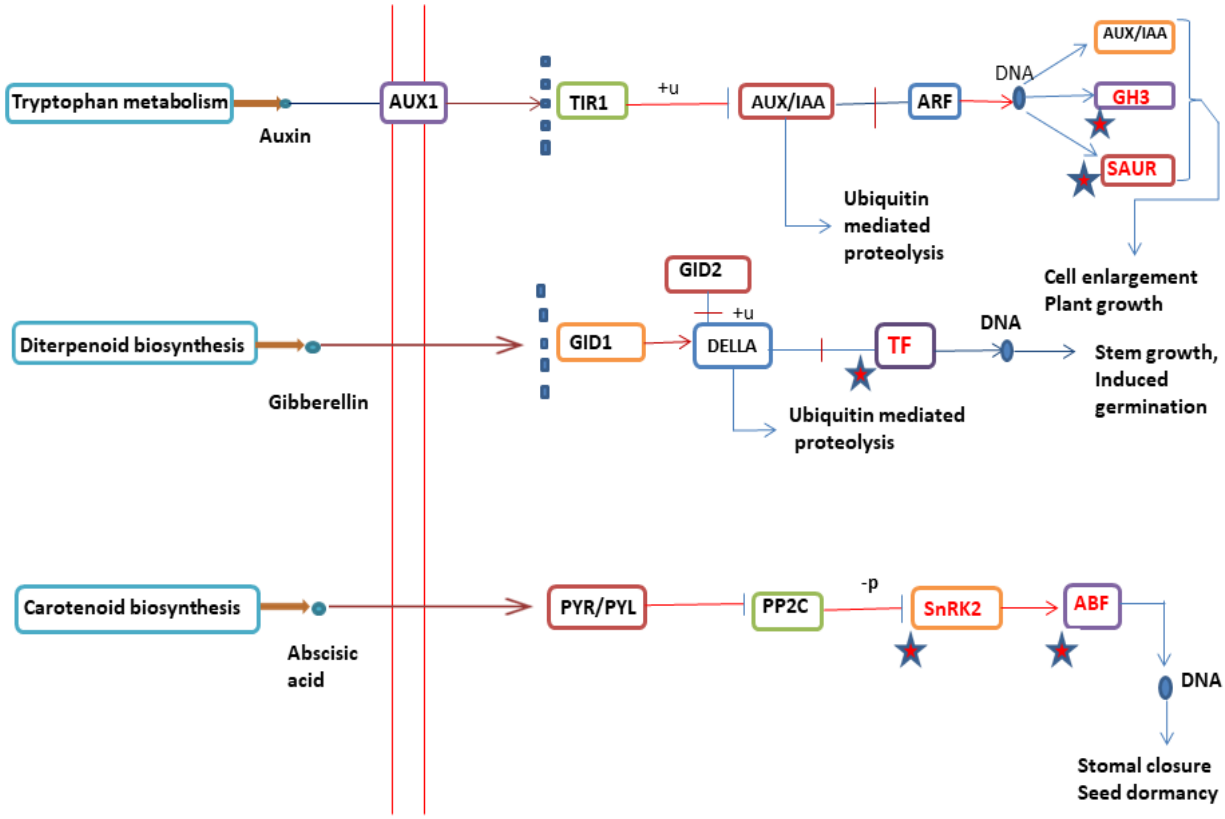


Fig 3.17. Plant hormone signal transduction pathway. [Transmembrane amino acid transporter family protein (AUX1) F-box/RNI-like superfamily protein (TIR1), AUX/IAA transcriptional regulator family protein, Auxin-responsive GH3 family protein (GH3), SAUR-like auxin-responsive protein family (SAUR), histidine-containing phosphotransfer factor (AHP), alpha/beta-Hydrolases superfamily protein 1(GID1), alpha/beta-Hydrolases superfamily protein 2(GID2), Transcription Factor (TF), Polyketide cyclase/dehydrase and lipid transport superfamily protein (PYR/PYL), Highly ABA-induced PP2C protein 2 (PP2C), sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK2), ABA-responsive element binding factor (ABF)]. The upregulated proteins are marked by asterisks.

In the exclusively treated group, other genes such as Lhcb1: PSII Light harvesting complex protein 1 ($p<0.032$), Lhcb2: photosystem II light harvesting complex protein 2 ($P<0.014$), Lhca1: chlorophyll a-b binding protein 6 ($P<0.022$) were found to be significantly expressed. All these genes code for proteins are required for the assembly of light harvesting chlorophyll protein complex in the thylakoid membrane (Fig 3.17).

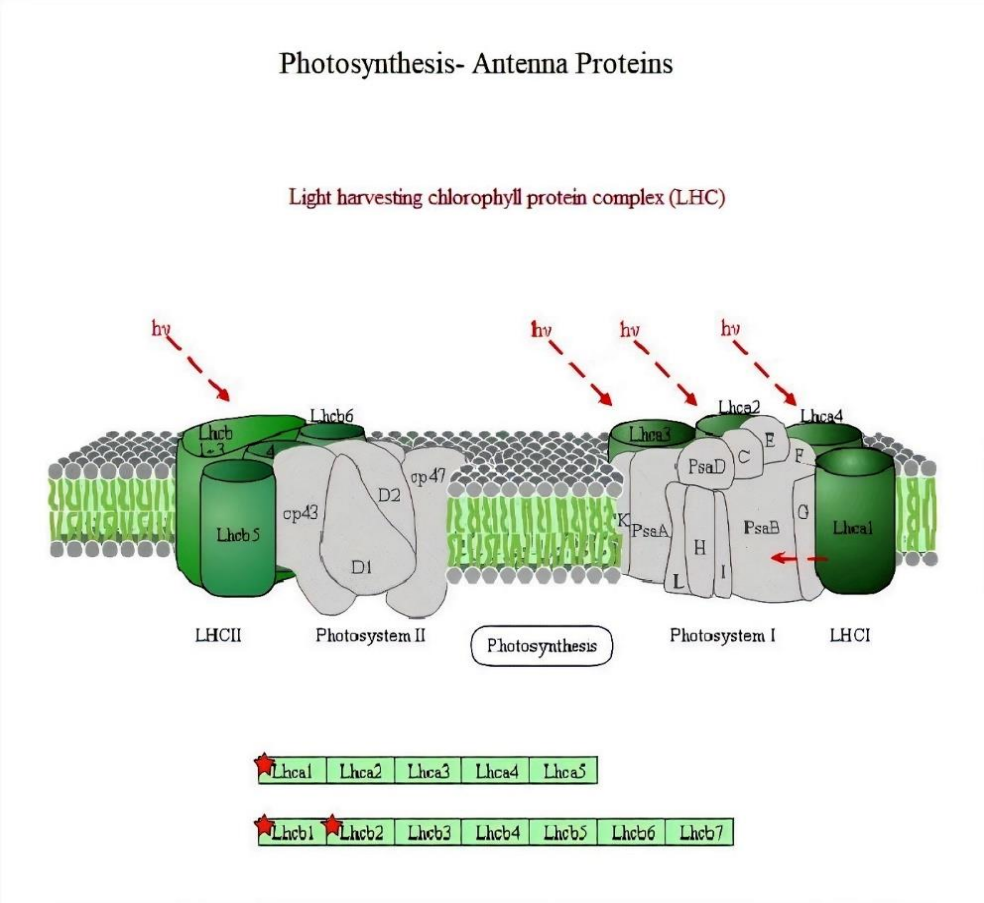


Fig 3.17. Antenna protein mediated photosynthesis in plant [Lhca1 (Chlorophyll a/b binding protein 6), Lhca2 (PSI type II chlorophyll a/b- binding protein), Lhca3 (PSI type III chlorophyll a/b- binding protein), Lhca4 (Light harvesting chlorophyll protein complex I subunit A4), Lhca5 (PSI light harvesting complex protein 5), Lhcb1 (PSII Light harvesting complex protein 1), Lhcb2 (PSII Light harvesting complex protein 2), Lhcb3 (Light harvesting chlorophyll b protein binding protein 3), Lhcb4 (Light harvesting complex of PS II subunit 4) Lhcb5 (Light harvesting complex of PS II subunit 5) Lhcb6 (Light harvesting complex of PS II subunit 6) Lhcb7 (Light harvesting complex of PS II subunit 7)]. The upregulated proteins are star marked.

3.4. Discussion

Excessive and indiscriminate use of inorganic fertilizers has dramatically increased the crop yield to meet the global food demand. However, such agricultural practice happens to be the

deleterious to the environment, particularly, the contamination of underground water, rivers, pond and the soil salinization, and thus questioning the sustainability of crop production (Phogat et al., 2014). Hence, the eco-friendly, organic and sustainable agriculture processes and technology are highly desirable (Esitken et al., 2005). Recent decade has witnessed a surge in the organic farming but the beneficial effects of organic farming are coupled with compromised crop yield and therefore, strategies to maintain agricultural productivity under reduced use of the chemicals in form of fertilizers and pesticides are highly warranted. The beneficial effects of PGPR on plant growth promotion and biocontrol of plant pathogens are well reported; however, their role on the improved utilization of chemical nutrients has not been thoroughly explored (Armada et al., 2014). Among various plant nutrients N is required in the greatest quantities for biosynthesis of amino acids, proteins, DNA, RNA, phytohormones and other secondary metabolites and thus plays significant role in increasing the productivity of major food crops (Chardon et al., 2012; Lassaletta et al., 2014). Hence, present investigation was carried out to study the effect of PGPR supplementation to the NF with varying input of N on plant performance. For achieving the objective, the mustard plants were treated with the NF having differential N inputs and two PGPR isolates *C. davisae* RS3 and *K. pneumoniae* RS26, employing both the general and statistical approaches and their effect on plant growth and yield were compared by monitoring morphological and biochemical parameters, and seed yield. The plants under NF treatment regime showing highest growth and yield was subjected to differential transcriptomics analysis using plants treated with optimum level of inorganic N as control.

In the general approach of treatment, mustard plants were grown under four different NF treatment regimes, i.e. N-appropriate without microbes (N^+PGPR^-), N-appropriate with microbes (N^+PGPR^+), N-deficit without microbes (N^-PGPR^-), N-deficit with microbes (N^-PGPR^+). The quantitative values for all the morphological parameters of N^+PGPR^+ and N^-PGPR^+ treated plants were comparable and were greater than that of plants treated with N^+PGPR^- or N^-PGPR^-. In case of biochemical parameters, though the level of chlorophyll a did not vary significantly between various NFs, chlorophyll b content was noticed to be higher in N^-PGPR^+ and N^+PGPR^+ plants as compared to N^+PGPR^- and N^-PGPR^- plants throughout the plant growth period. Chlorophyll b is an antenna chlorophyll and its binding to the antenna protein is crucial for correct assembly of the thylakoid membrane (Hoober et al., 2007). Chlorophyll b also has a major impact on lateral mobility and diffusion of membrane molecules, light harvesting and thermal energy dissipation processes, electron transport, and repair processes in grana (Hoober et al., 2007). The higher level of chlorophyll b in the shoot of N^-PGPR^+ plants was found to be correlated with their greater carbohydrate content compared to that of N^+PGPR^- and N^+PGPR^+ plants. The effect of PGPR application on enhancement of plant carbohydrate content has already been shown in earlier studies. The carbohydrate content of *Amaranthus hypochonariacus* increased by 49 and 34.6% on application of three and two *Bacillus* strains, respectively, when compared with PGPR untreated plants (Pandey et al., 2018).

Similarly, a significant increase of plant total carbohydrate and mineral contents of wheat was recorded upon PGPR (*Azotobacter* and *Rhizobium*) treatment (Al-Erwy et al., 2016).

In higher plants, ammonia either absorbed from soil, or produced via nitrate reduction and biological nitrogen fixation, is mainly assimilated to glutamine by the activity of GS and therefore, GS activity can serve as a measure of ammonia uptake and assimilation (Singh and Ghosh, 2013). The activity of GS was increased in shoot of N^+PGPR^+ and N^-PGPR^+ plants up to 15 days of growth and then declined. The shoot, especially leaves act as sink for N during vegetative stage (Xu et al., 2012) and enhanced GS activity indicates the role of bacterial inoculum in improving uptake and assimilation of ammonia. The plant uptake of ammonium produced by PGPR in fact requires alteration in bacterial N-metabolism so that N is excreted rather than incorporated into microbial biomass. Previous research works have shown that N metabolism is significantly altered during rhizobium-legume symbiosis with ammonia-assimilation being effectively shut down in bacterium. Inactivation of ammonium assimilation maybe accomplished via an unknown and probably plant-regulated post translational modification of GS (Bravo and Mora, 1988; Patriarca et al., 2002). The application of NF supplemented with *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26 also enhanced the protein content of mustard plant with a significantly greater level in N^-PGPR^+ treatment. Increased plant protein content might be correlated with the N fixing ability of both the PGPR strains; the fixed ammonia in turn be utilized for amino acids and protein synthesis in the plant as evidenced by Kumar et al., 2015. As observed in this study, the protein content of bean seeds was found to increase by about 12% in presence of PGPR in growth medium (Stefan et al., 2013). In a research conducted by Kang et al (2012), noticed enhanced amino acid and protein content of cucumber plants due to the application of PGPR. The results of general approach thus showed that among the four different NFs used, N^-PGPR^+ formulation with sub-optimum level of N and the two PGPR, supported maximum growth and yield as reflected by higher root and shoot lengths, root and shoot fresh weight, carbohydrate and protein contents, number of siliqua $plant^{-1}$, number of seed $siliqua^{-1}$, 100 seed weight, and seed yield $plant^{-1}$. A study done by Akhtar et al. (2009) on wheat showed that application of PGPR in combination with recommended dosages of inorganic fertilizer resulted in maximum increases in yields of grain and straw. The N^-PGPR^+ and N^+PGPR^+ treatments differed only with respect to the concentration of N with the former having sub-optimum level of N. Previous studies have shown that plant growth is not only dependent on N forms but also on N concentration (Hageman, 1984, Walker et al., 2001). The research work of Wang et al. (2011) showed reduction in grain yield in presence of excess soil N. It is also known that ammonium, the most important source of N for plant growth, promote plant growth at lower concentration of external supply, however, its higher external supply causes toxicity with inhibition of root and shoot growth, leaf chlorosis and ionic imbalances, disturbance of pH gradients across plant membranes, or oxidative stress (Liu and Wiren, 2017). In this study mustard plants of N^-PGPR^+ and N^+PGPR^+ treatment groups were treated with NFs containing ammonia at suboptimum and optimum concentration, respectively, however, the conditions might alter in presence of N

fixing PGPR. A relatively lower growth and yield of mustard plants under N^+PGPR^+ than those under N^-PGPR^+ could be related to the enhancement in N supply to the plants above the optimum level due to the N-fixing ability of both the PGPR supplements to the NF. Recently, a research work by Xia et al. (2017) found that in soyabean (*Glycine max* L.) plant, root nodulation and nitrogenase activity were accelerated by low N concentrations (<50 mg L^{-1}) and suppressed by high concentrations of nitrogen (>50 mg L^{-1}). Although nitrate is the primary N source for mustard plant, ammonium ion alters root system architecture by inhibiting root elongation and by stimulating lateral root branching and root hair growth and becomes the preferable N source. Accordingly, a strictly regulated ammonium uptake system must exist in the plant, including abundant ammonium transporters and effective mechanisms regulating their activity in *Brassica sp.* (Zhu et al., 2018).

The improved performance of plants under N^-PGPR^+ and N^+PGPR^+ treatment formulations as compared to that of N^-PGPR^- and N^+PGPR^- groups might be correlated with the IAA production, phosphate solubilisation and zinc solubilisation properties of the supplemented PGPR. Earlier studies have shown that the capability of the PGPR strains to solubilize phosphate and zinc; produce ammonia and phytohormones; and enhance nutrient availability and uptake especially of N, which has significant role in plant growth promotion during vegetative growth (Metay et al., 2015; Kiani et al., 2016). As observed in this study, previous research works have reported the PGPR mediated enhancement in flowering, fruit development and seed yield of *Brassica* (Pattison et al., 2014; Ahmad et al., 2016). Also the supplementation of PGPR along with inorganic N fertilizer resulted in an improvement of plant health and crop yield comparable or greater than that observed when general quantities of inorganic N is applied.

Further, the statistical approach of CCRD based RSM was implicated in optimization of NF for plant treatment with respect to differential-N and PGPR inputs for enhanced plant growth and yield. Successful application of RSM to improve plant growth and yield has been reported in various studies (Peng et al., 2014; Naili et al., 2018). However, research on statistical optimization of plant growth and yield through optimization of bioinoculation parameters under different N concentration are still few in the literature. In the present study, when compared with the general method, RSM design based treatment of plants (Fig 3.10) resulted in significantly greater growth and yield of mustard plant as evidenced by relatively greater fresh weight of root and shoot, root length, stem length, carbohydrate content of root and shoot, protein content of root and shoot, GS activity of root and shoot, Chlorophyll a and b contents, seed yield (1.24g, 2.05g, 8.78 cm, 25.45cm, 8.67 mg g^{-1} FW, 16.12 mg g^{-1} FW, 543.18 mg g^{-1} FW, 924.76 mg g^{-1} FW, 19 µmole g^{-1} FW, 211 µmole g^{-1} FW, 2.7 mg g^{-1} FW, 1.32 mg g^{-1} FW and 8.10 g $plant^{-1}$ respectively as compared to plants in N^-PGPR^+ treatment). Few reports have suggested the improvement in plant growth potential of PGPR, when they were applied using RSM technique (Rajput et al., 2013; Desale et al., 2014). Similarly, Naili et al. (2018) also found that application of the two PGPR strains, *Piscibacillus salipiscarius* E5 and *Halomonas*

sp. G11, as bioinoculant to treat wheat plant under salt stress using Box Benker design (BBD) design based RSM technique resulted in improvement of the plant growth parameters and yield.

Mustard plants treated with NFs showing highest yield was subjected to transcriptomic analysis in order to gain an insight into the role of NF in plant growth promotion. Transcriptome analysis of mustard plant root identified 25,088 genes expressed both in control and treatment groups, whereas 357 genes were expressed exclusively in PGPR treated plant and 351 genes were exclusively expressed in control plants. The treatment of RSM optimized NF to the plants resulted in downregulation of 690 genes (690), whereas 556 genes were upregulated. Similar results were also obtained by Hao et al. (2016), where transcriptomes profile of *Arabidopsis* leaves treated with volatiles emitted by *Bacillus amyloliquifaciens* showed more downregulated genes than upregulated genes. In this present study, genes related to extracellular region, cell junction, protein containing complex, catalytic activity, binding activity, transporter activity, molecular transducer activity, nutrient reservoir activity, cellular process, developmental process, metabolic process, reproductive process, response to stimulus, signaling, localization, growth, detoxification, carbon utilization were significantly upregulated, whereas genes involved in transcriptional regulator activity, antioxidant activity, metabolic processes, cellular component organization or biogenesis, biological regulation, multi-organism process, positive and negative regulation of biological process, signaling were found to be downregulated significantly in PGPR treated plants. Moreover, the upregulated genes were found to be associated with glyoxylate and dicarboxylate metabolism, carbohydrate metabolism (glycolysis, pentose and gluconate interconversion), lipid metabolism (fatty acid biosynthesis, steroid biosynthesis), environmental adaptation (plant pathogen interaction, circadian rhythm, thermogenesis), plant secondary metabolites (thiamine, amino sugar, nucleotide sugar).

The results of transcriptomics analysis showed enhanced expression of several genes directly and indirectly associated with improved plant growth and development in the roots of treated group of plants, like genes for ammonium and amino acid transporters, ammonium and amino acid biosynthesis. biotic and abiotic stress tolerance, auxin signaling, light reaction of photosynthesis, antioxidant pathways, promotion of flowering, lipid storage proteins etc.

Nitrogen acquisition is a fundamental process for living beings, including plants in crops. In non-legume plants the acquired N is reduced to amino acids in roots or photosynthetically active leaves. The amino acids representing the main long distance transport form of N move from source to sink that rely on the organic forms of N for growth and development. Developing leaves and roots are the major N-sink during the vegetative growth phase, whereas flowers and fruits ate the major sink during reproductive stage (Masclaux-Daubresse et al., 2010; Tegeder and Masclaux-Daubresse, 2017). This sequence of transport of N from soil to source to sink requires plasma membrane localized transport proteins in the source and sink. A number of organic and inorganic N transporters with different substrate affinities and specificities have been identified in roots. They are reported to regulate root uptake and root to shoot transport

(Fan et al. 2017). This investigation showed significant increase in expression of ammonium and amino acid transporter mRNAs in the roots treated group of mustard plant in comparison to control, which in turn supported greater growth and yield of former. The transporter expression was also found to be coupled with the upregulation of genes encoding various amino transferases or transaminases that catalyze the interconversion of amino acids and oxoacids by transfer of amino groups.

Nitrate reductase catalyzes the synthesis of ammonia from nitrate through nitrite reductase and the enzyme is reported undergo overexpression in presence of light and nitrate (Aslam et al. 1987). The incorporation of N from nitrate first requires its acquisition from the medium by specific transporters, which are responsible for the sensing, uptake, storage and distribution of nitrates among plant tissues. Although NO_3^- was not added in the growth medium, the enhanced expression of nitrate reductase mRNA in the root of mustard plant by 1.5569 log 2 fold indicate nitrifying ability of the supplemented PGPR in the NF used for treated group of plants. In previous study, few *Klebsiella* species are shown to have heterotrophic nitrifying and aerobic denitrifying abilities (Feng et al. 2018).

WRKY is a major transcription factor family with several numbers like 109 in rice and 74 in *Arabidopsis*. They are reported to contain ≈60 amino acid long four-stranded β-sheet WRKY DNA binding domain/s (DBD) and Zinc-finger motifs. WRKYs act through various interconnecting signaling networks to regulate multiple responses simultaneously whether it is biotic, abiotic, or physiological (Banerjee and Roychoudhury, 2015). They have been shown to confer resistance to several biotic stress caused by multiple bacterial or fungal agents. Present study revealed enhanced expression of WRKY11 and reduced expression of WRKY18 in the roots of treated group of plants. The research work of Wu et al (2009) showed that overexpression of OsWRKY11under the control of HSP101 promoter leads more chlorophyll content and low leaf wilting in rice under drought stress. Similarly, ectopic expression of WRKY11 in *Arabidopsis* thaliana contributed to osmotic tolerance to osmotic stress (Liu et al. 2011). In a report of Chen et al. (2010) it was demonstrated that the WRKY18 and WRKY60 mutants are more tolerant to osmotic stress. WRKY18 and WRKY60 are positive regulator of ABA and stress conditions.

The expression of mRNA for Lysine/Histidine transporter was found to increase by 3.84131 log 2 fold in treated plant group compared with control plants. The ectopic overexpression of a gene encoding cationic amino acid transporter (CAT1) has been shown to improve the disease resistance to a hemibiotrophic bacterial pathogen in *Arabidopsis* via a constitutively activated salicylic acid pathway (Yang and Ludewik, 2014). In another study, *P. syringae* infection to Arabidopsis led to over expression of Lysine/Histidine and CAT1 transporters (Navarova et al. 2012). The disruption of Lysine/Histidine transporter gene function has been shown to markedly inhibit rice growth (Wang et al. 2019). Hence, upregulation of these transporter mRNAs in treated group plants might contributed to their improved growth through

development of resistance against plant pathogens. Similarly, UDP-glycosyltransferase enzymes (UGTs) are known to function in detoxification and enhance *Fusarium* head blight (FHB) resistance by glycosylating deoxynivalenol (DON) into DON-3-glucoside (D3G). He et al. (2018) performed an expression analysis of online universal microarray data in certain tissues at different developmental stages to understand the roe of UGTs the life cycle of *Triticum* plant. He et al. (2000) reported that in the microarray results probes that specifically match 61% of the identified wheat UGTs were found and during the whole life cycle of the plant most of these genes have been expressed at least in a certain tissue. The upregulation of phenylalanine metabolism (L-phenylalanine ammonia-lyase (PAL^{+}) in our study also supported by the research exhibited by Hyun et al., 2011 where higher level of PAL expression was found to be coupled with reduced susceptibility of plant against fungal pathogen. Also the induction of plant defense mechanism genes correlated with carbohydrate and fatty acid metabolism, and chlorophyll biosynthesis clearly suggesting beneficial effect of PGPR treatment in improved plant growth (Zhang et al., 2009).

In plants, aerobic reactions like respiration and photosynthesis lead to production of reactive oxygen species (ROS). These ROS, such as hydroxyl radicals, superoxide radicals, or hydrogen peroxide (H_2O_2), can damage biological molecules, including proteins, nucleic acids and lipids. The plant ROS levels significantly increase during environmental and biotic stress conditions. Plants have developed non-enzymatic and enzymatic systems to combat the oxidative damage caused by these ROS. Glutathione peroxidases (Gpxs) are involved in reduction of ROS peroxides and they express in several plant organs and sub-cellular locations. The abundance of some Gpxs has been reported to modify in plants subjected to environmental constraints, generally increasing during water deficit, fungal infection, and metal stress, and decreasing during photooxidative stress, showing that Gpx proteins are involved in the response to both biotic and abiotic stress conditions (Navrot et al. 2006). A significantly higher level of expression of Gpx in the roots of treated group of plants (1.872908 log 2 fold change) indicate the development of stress tolerance of the plants on treatment with NF with reduce N input and PGPR.

Auxin has been recognized as a major hormone shaping plant architecture. It influences stem elongation and regulates the formation, activity, and fate of meristems. According to Chapman and Estelle (2009), the present knowledge on the auxin signaling pathway has greatly extended over the past two decades, which has resulted in the establishment of a core model for auxin signal transduction. Auxin is perceived by co-receptor complexes formed by F-box Transport Inhibitor Response1/Auxin Signaling F-Box (TIR1/AFB) and Auxin/Indolacetic3 Acid (Aux/IAA) proteins (Gallavotti, 2013). The stable interaction between auxin, TIR1/AFB, and Aux/IAA proteins triggers the degradation of the transcriptionally repressive Aux/IAA proteins (Tan et al., 2007). Their subsequent ubiquitanation by the 26S proteasome ensures that the Auxin Response Factors (ARFs) (transcriptional regulators) which forms heterodimers with the Aux/IAAs, can induce transcription of early auxin-responsive genes and therefore results in developmental response to auxin. Some well-known early auxin-responsive genes include

GH3, *SAUR* genes, and the *Aux/IAA* genes themselves. SAURs (Small Auxin Upregulated Proteins) are small proteins that are upregulated shortly after auxin induction. Recent studies have shown that these are important for internode and stem elongation in *Arabidopsi*s. When fused to GFP (Green Fluorescent Protein), but not to a shorter epitope tag such as hemagglutinin (HA), SAURs cause elongation of stems. Plants which express SAUR–GFP proteins have shown higher basipetal auxin transport, indicating the direct or indirect effects on auxin transport. Loss-of-function artificial microRNA lines show shorter hypocotyls and stamen filaments, suggesting that *SAUR* genes promote auxin-induced growth (Chae *et al.*, 2012). Fig 3.17 depicting the plant hormone signaling pathway. In the present study, five genes associated with the phytohormone signal transduction pathway were found to be elevated. They encoded Auxin-responsive GH3 family protein (GH3), SAUR-like auxin-responsive protein family, ABA-responsive element binding factor (ABF), Sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK2), and basic-leucine zipper (bZIP) transcription factor family protein.

Light-harvesting complexes (LHC) are a series of membrane proteins which absorb sunlight (Roach and Krieger-Liszkay, 2014). LHCII is the most abundant of these pigment-protein complexes. The LHCs have a dual function. In low light conditions, they absorb solar energy and transfer the excited energy to the reaction center. In high light conditions, they additionally play a role in photoprotection by dissipating the excess absorbed energy in the form of heat (Rochaix, 2014). There is a regulatory interaction between light harvesting, electron transport and carbon assimilation. According to Horton, 2012, active regulatory mechanisms controlled both the activity of the thylakoid processes producing ATP and NADPH and the stromal reactions of carbon assimilation that consume them. Fig 3.17 showing antenna protein mediated photosynthesis in the plant. In the present study, three genes namely Lhca1 (Chlorophyll a/b binding protein 6), Lhcb1 (PSII Light harvesting complex protein 1), Lhcb2 (PSII Light harvesting complex protein 2) were upregulated. In plants, photosynthesic genes are present in both the nuclear and plastid genomes (Martin et al. 2002). However, genes for LHC proteins and pigment biosynthesis pathways are found in the nuclear genome and those genes encoding photosystem core subunits are mainly in the plastid genome. Therefore, chloroplast biogenesis depends on close cooperation between the nuclear and plastid genomes. Interestingly, in one of the findings, the reduced expression of photosynthetic genes was observed in roots of the plants under inorganic phosphorous deficiency condition (Li et al., 2010; Kang et al., 2014). Several microarray analyses indicated that the expression of photosynthetic genes was downregulated in the inorganic phosphorous deficit plants (Wu et al., 2003; Li et al., 2010; O'Rourke et al., 2013). On contrary, in the present study, it was found that the expressions of LHC genes were elevated in the root of RSM optimized PGPR supplemented NFs treated mustard plant. This may be due to the presence of PGPR supplemented NF treatment which helps the plant with soluble phosphorous. Because root is thought to be a non-photosynthetic organ, the biological relevance of this phenomenon has received little attention and needs to be explored.

Table 3.18
Details of the genes upregulated and downregulated in heat map

Gene code	Gene Name	FPKM_ Control	FPKM_ Treated	log2 Fold change	Role
Bra017702	Lys/His Transporter 7	77.55034	1108.222	3.84131	Growth and yield
Bra001626	Ammonia transporter/ methylammonium transmembrane transporter/ water channel	13.4591	433.321	3.30825	Transports ammonia
Bra016669	Pleiotropic Drug Resistance 7	21.0473	196.82	3.22517	Pathogen resistance and new role for the ATP-binding cassette transporter family.
Bra006196	Auxin-Responsive GH3 Family Protein	30.8032	334.429	1.67216	Regulates auxin-response genes and Induced Systemic Resistance
Bra018793	Glycosyltransferase Family Protein	6.34185	47.4314	2.90287	Protein modification
Bra034609	Quercetin 7-O-Glucosyltransferase	17.77605	148.0902	2.62863	Transfer a glucosyl group from UDP-glucose to the 7-hydroxy group of a quercetin molecule.
Bra030620	UDP-Glucosyl Transferase Family Protein	16.3827	101.174	2.6266	Detoxification and subsequent elimination of xenobiotics
Bra032019	NADP-Dependent Oxidoreductase	86.3818	506.79	2.55259	Pivotal role in central metabolic pathways
Bra036217	Chorismate Mutase 1	36.4185	185.928	2.352	Biosynthesis of tyrosine and phenylalanine
Bra036259	Glutathione S-Transferase F3	46.4977	229.454	2.30297	Detoxify xenobiotics
Bra021934	WRKY35 transcription factor	14.18726	59.9089	2.24933	Pollen development, ending seed dormancy
Bra000122	Mildew Resistance Locus O 12	4.39318	49.4435	2.14595	Resistance against fungus
Bra021716	INH3	30.5168	129.002	2.07972	Inhibits Protein phosphatase 1 activity
Bra005595	Galactosyltransferase Family Protein	15.7884	62.77	1.99121	Protein modification
Bra013634	Monooxygenase	109.884	422.004	1.94127	Detoxification, defence against pathogen and auxin synthesis
Bra010231	Wrky11 transcription factor	67.2396	257.516	1.93728	Defense against Pathogen
Bra038537	Aminotransferase Class I And II Family Protein	87.3825	329.009	1.91271	Amino acid biosynthesis and catabolism, photorespiration, and vitamin biosynthesis, as well as carbon and nitrogen shuttles
Bra005818	myb Family Transcription Factor	84.2069	208.544	1.85093	Regulatory roles in developmental processes and defense responses
Bra019570	Mitochondrial Phosphate Transporter	14.019	49.4501	1.81859	ATP production
Bra038807	CTP Synthase	0.618186	200.23	1.79267	Gene transcription and for nucleic acid synthesis during cell division
Bra022060	Ubiquitin Thiolesterase	0.534018	1.85014	1.79267	De-ubiquitinating proteases helps in shoot development
Bra001874	Seed Storage/Lipid Transfer Protein (LTP) Family Protein	85.2778	271.346	1.66989	Pathogenesis-related proteins, seed development

Bra014354	Disease Resistance Response	147.618	459.193	1.63723	Recognizing specific pathogen effector molecules produced during the infection process.
Bra011755	Monooxygenase	14.694	308.694	1.62953	Detoxification, defence against pathogen and auxin synthesis
Bra011741	APETALA 2 Transcription Factor	31.9769	695.8511	1.58377	Seed development
Bra024915	Phototropic-Responsive NPH3 Family Protein	3.52152	9.69604	1.4612	Necessary for phototropism
Bra007225	Amino Acid Transporter Family Protein	35.7847	197.3936	1.44448	Plant growth and development
Bra017210	Phenylalanine Ammonia-Lyase	372.985	931.505	1.32044	Synthesis of a variety of polyphenyl compounds
Bra004810	Methylene Tetra Hydrofolate Reductase 2	58.688	116.728	0.992015	Biosynthesis of methionine
Bra033741	Inorganic Phosphate Transmembrane Transporter	10.4559	20.2316	0.952294	Transport of inorganic phosphate (Pi) through plant membranes
Bra009799	Flowering Promoting Factor 1	29.2383	236.6876	0.931311	Modulates the competence to flowering of apical meristems
Bra011781	Growth Regulating Factor 2	14.7185	559.6431	0.907135	Development of gynoecia and anthers.
Bra035211	Glutathione Peroxidase	1513.6	2771.93	1.872908	ROS scavenging
Bra011724	Aldose 1-Epimerase Family Protein	52.8873	94.4142	0.836083	Carbohydrate metabolism
Bra011778	N-Acetyltransferase (GNAT) Family Protein	20.3158	31.4497	0.63044	Involved in many cellular processes such as transcriptional activation, gene silencing, cell cycle regulation, DNA replication/repair and chromosome assembly
Bra003670	Nitrate Reductase 1	222.5835	1133.2228	1.5569	Catalyzes the first step in reduction of nitrate N to organic forms
Bra011804	Transmembrane Transporter	13.0409	18.4849	0.503302	Plants take up and use water and nutrients
Bra022345	MAP Kinase Substrate 1	124.979	175.025	0.485878	Phosphorylate their substrates and this post-translational modification (PTM) contributes to the regulation of proteins.
Bra004134	NADP+ Isocitrate Dehydrogenase	155.119	206.974	0.416071	Production of 2-oxoglutarate for ammonia assimilation, biosynthesis of glutamate.
Bra036409	Copper Uptake Transmembrane Transporter	46.679	38.7333	-0.2692	Uptake of copper ions
Bra018695	Cell Wall / Vacuolar Inhibitor of Fructosidase 1	582.077	182.883	-1.67029	Regulates ABA response
Bra011299	WRKY18 Trancription Factor	283.961	52.7774	-2.4277	Positive regulators of plant ABA
Bra028896	Pyruvate Decarboxylase	1218.52	154.71	-2.97749	Switch to fermentative pathway
Bra017509	Senescence-Associated Protein-Related	526.726	48.9714	-3.42704	Degradative processes leading to the remobilization of nutrients and eventual leaf death.
Bra011294	Glutamine Dumper 1	133.68	28.6111	-2.22414	Overexpression leads to free amino acid levels accumulation, plant size decrease

In conclusion, the supplementation of two potent PGPR (*Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26) under low N input ($N^{-}PGPR^{+}$) with general approach has improved the growth and seed yield of mustard plants. The formulation optimized with RSM based approach proved to be fruitful in enhancing the overall morphological, physiological and biochemical performances of the plants. Comparative transcriptomic analysis of plants treated with RSM optimized NF as treated group and those treated with appropriate level of N without PGPR as control group revealed significant alterations in plant gene expression. Application of RSM optimized NF to the plant resulted in upregulation of several genes encoding proteins or enzymes to be directly associated with plant productivity, like nitrate reductase, ammonia transporter, amino acid transporter family protein, inorganic phosphate transmembrane transporter, flowering promoting factor 1, seed storage/lipid Transfer Protein (LTP) family protein, mitochondrial phosphate transporter. Furthermore, several gene products which indirectly affect plant performances by giving resistance against biotic and abiotic stress were also upregulated, Lys/His transporter 7, UDP-glucosyl transferase family protein, glutathione S-transferase F3, mildew resistance locus O12, INH3, monooxygenase, disease resistance response and pleiotropic drug resistance 7, phenylalanine ammonia-lyase, WRKY35 transcription factor, NADP-dependent oxidoreductase, chorismate mutase 1, CTP synthase, growth regulating factor 2, glutathione peroxidase, transmembrane transporter and $NADP^{+}$ isocitrate dehydrogenase (Table 3.18). Metabolic pathway analysis portrayed the expression of the genes related to plant hormones signaling and light harvesting genes were also upregulated. Thus, the RSM approach can be a promising tool for designing the nutrient formulations containing PGPR and reduced inorganic fertilizer levels for improved plant growth and yield in a cost-effective manner, and thus can put a brick in the development of sustainable agriculture.

SUMMARY & CONCLUSION

The research concluded with the following outcomes:

1. Among the 82 rhizospheric isolates altogether, twenty-seven isolates showed positive growth on Asbhy's N-free medium, a differential media for screening PGPR with potential biological N_2-fixation ability.
2. Further, N_2-fixer isolates were screened qualitatively and quantitatively for other PGP traits and all of them exhibited PGP traits viz. ammonia production, inorganic phosphate solubilisation, organic phosphate solubilisation, zinc solubilisation, IAA production, ACC deaminase production.
3. Majority of PGPR belonged to the class gamma-proteobacteria and few were members of class betaproteobacteria and phylum Firmicutes.
4. Two bacterial isolates (RS3 and RS26) showed significantly higher production of ammonia, auxin and ACC deaminase; and solubilisation of Zinc and phosphate. They were selected as the two most potent PGPR strains using principal component analysis (PCA) of their qualitative PGP traits.
5. These two strains RS3 and RS26 were negative for hemolysin production in sheep blood agar medium. The strains didn't show antagonistic effect indicating their compatibility with each other and hence, selected for further study.
6. The phylogenetic analysis based on 16S rRNA gene sequence analysis identified the bacterial isolates RS3 and RS26 as *C. davisae and K. pneumoniae* and therefore, names as *C. davisae* RS3 and *K. pneumoniae* RS26, respectively.
7. PGPR strains RS3 and RS26 are used for designing nutrient formulations (NF) by general and statistical (RSM) method, for application to mustard plants.
8. In general method, the mustard plants were divided into four different treatment regimens based on NF treatments, namely, N-appropriate without microbes, N-appropriate with microbes, N-deficit without microbes and N-deficit with microbes. Plants were analysed for morphological, biochemical and physiological parameters at 15, 45 and 75 days after treatment (DAT). Among the various NF treatments, plant under N^-PGPR^+ treatment regimen showed significantly higher plant growth and seed yield parameters. For example, seed yield of N^+PGPR^-, N^+PGPR^+, N^-PGPR^-, N^-PGPR^+ plants were 3.98, 4.08, 3.22, and 5.76 g $plant^{-1}$, respectively.
9. Plants treated with RSM optimised NF with conditions 0.50 mM N, 50% v/v of Strain 1(RS3) and 50% v/v of strain 2 (RS26) were found to have better growth performance and productivity than N^-PGPR^+ plants. Consequently, at 75 DAT, N^-PGPR^+ and RSM optimized NF treated plants showed carbohydrate content, protein content and seed yield of 12.17 mg g^{-1} FW, 675.89 mg g^{-1} FW, 5.76 g $plant^{-1}$, and 16.12 mg g^{-1} FW, 924.76 mg g^{-1} FW, 8.10 g $plant^{-1}$, respectively.
10. Mustard plants treated with RSM optimized NFs showing highest yield was subjected to differential transcriptomic analysis (DGE) in order to gain an insight into the role of

NF in plant growth promotion. Mustard plant treated with N appropriate (5 mM N) NF without PGPR served as control.

11. The DGE results showed that 25,088 protein coding genes were expressed in both control and treated group of plants, where as 357 genes were exclusively expressed in treated group only, 351 genes were exclusively expressed in control group of plants only. The expression level of total 556 genes were found to be up regulated and 690 genes were found to be downregulated as compared to the control group
12. It has been found that more than 10% of the genes such as genes from organelle, cell, cellular part, membrane assembly were down regulated and designated as a part of cellular component, whereas genes involved with catalytic activity, protein binding, transcriptional regulator activity, antioxidant activity, metabolic processes were downregulated as a part of molecular function and gene cluster of biological process such as cellular component organization or biogenesis, biological regulation, multi organism process, positive and negative regulation of biological process, signalling were found to be down regulated significantly in PGPR treated plants.
13. The genes that were downregulated and upregulated in the treated group with respect to control group are shown in the Fig 3.15 and Fig 3.16 respectively. Among the cellular components and biological processes, the expression of genes related to organelle, extracellular region part, localization, cell proliferation, detoxification, carbon utilization was increased, whereas genes encoding proteins for nucleiod and rhythmic processes were found to be downregulated. The results of transcriptomics analysis showed enhanced expression of several genes directly and indirectly associated with improved plant growth and development in the roots of treated group of plants, like genes for ammonium, nitrate and amino acid transporters, ammonium and amino acid biosynthesis. biotic and abiotic stress tolerance, auxin signaling, light reaction of photosynthesis, antioxidant pathways, promotion of flowering, lipid storage proteins, resistance to pathogens etc. Expressions of mRNA of some genes related to copper uptake transmembrane transporter, ABA responses, senescence associated proteins, accumulation of free amino acids etc. were downregulated.
14. The functional annotations of the genes were carried out against the curated KEGG gene data base using KAAS (KEGG automatic annotation server). The KEGG ontology (KO) database of plants was used as the reference for pathway mapping. Two pathways related to plant hormone signalling and antenna protein mediated photosynthesis were generated which potrayed the expression of the genes related to phytohormone signaling and light harvesting genes were also upregulated. Five genes associated with the phytohormone signal transduction pathway were found to be elevated. They encoded Auxin-responsive GH3 family protein (GH3), SAUR-like auxin-responsive protein family, ABA-responsive element binding factor (ABF), Sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK2), and basic-leucine zipper (bZIP) transcription factor family protein and three genes of antenna protein mediated photosynthesis namely Lhca1 (Chlorophyll a/b binding protein 6), Lhcb1 (PSII Light harvesting complex protein 1), Lhcb2 (PSII Light harvesting complex protein 2) were upregulated.
15. Application of RSM optimized NF to the plant resulted in upregulation of several genes encoding proteins or enzymes to be directly associated with plant productivity, like

nitrate reductase, ammonia transporter, amino acid transporter family protein, inorganic phosphate transmembrane transporter, flowering promoting factor 1, seed storage/lipid Transfer Protein (LTP) family protein, mitochondrial phosphate transporter. Furthermore, several gene products which indirectly affect plant performances by giving resistance against biotic and abiotic stress were also upregulated, Lys/His transporter 7, UDP-glucosyl transferase family protein, glutathione S-transferase F3, mildew resistance locus O12, INH3, monooxygenase, disease resistance response and pleiotropic drug resistance 7, phenylalanine ammonia-lyase, WRKY35 transcription factor, NADP-dependent oxidoreductase, chorismate mutase 1, CTP synthase, growth regulating factor 2, 1, glutathione peroxidase, transmembrane transporter and $NADP^+$ isocitrate dehydrogenase etc.(Table 3.18).

16. RSM approach can be a promising tool for designing the nutrient formulations containing potent PGPR *Cedecea davisae* RS3 and *Klebsiella pneumoniae* RS26 and reduced inorganic fertilizer levels for improved plant growth and yield in a cost effective manner, and thus can put a brick in the development of sustainable agriculture.

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Appendix A

LIST OF ABBREVIATIONS

ACC	1-Aminocyclopropane-1-Carboxylic Acid
ABF	ABA-responsive element binding factor
ABA	Abscisic Acid
AE	Agronomy Efficiency Of Fertilizer N
α	Alpha
GID1)	alpha/beta-Hydrolases superfamily protein 1
GID2	alpha/beta-Hydrolases superfamily protein 2
ANOVA	Analysis On Variance
ANR	Apparent N Recovery Rate
ANFA	Asbhy's N-Free Agar
ANFL	Asbhy's N-Free Liquid
APX	Ascorbate Peroxidase
GH3	Auxin-responsive GH3 family protein
β	Beta
BNF	Biological Nitrogen Fixation
BP	Biological Process
BSA	Bovine Serum Albumin
CAT	Catalase
CFE	Cell Free Extract
CC	Cellular Component
CCRD	Central Composite Rotatable Design
Lhca1	Chlorophyll a/b binding protein 6
CFU	Colony Forming Unit
CEE	Crude Enzyme Extract
CKs	Cytokinins
DAT	Days After Treatment
^{o}C	Degree Celcius
DCP	Dicalcium Phosphate
DGE	Differential Gene Expression
EtBr	Ethidium Bromide
ePGPR	Extracellular PGPR
(TIR1),	F-box/RNI-like superfamily protein
FW	Fresh Weight
γ	Gamma
GO	Gene Ontology
Ga	Gibberellins
GOGAT	Glutamine Oxo-Glutarate Amino Transferase
GS	Glutamine Synthetase
PP2C	Highly ABA-induced PP2C protein 2
AHP	histidine-containing phosphotransfer factor
HCN	Hydrogen Cyanide

IAA	Indole-3-Acetic Acid
ISR	Induced Systemic Resistance
iPGPR	Intracellular PGPR
JNFb	Jacob's N-Free Broth
Lhcb3	Light harvesting chlorophyll b protein binding protein 3
Lhca4	Light harvesting chlorophyll protein complex I subunit A4
Lhcb4	Light harvesting complex of PS II subunit 4
Lhcb5	Light harvesting complex of PS II subunit 5
Lhcb6	Light harvesting complex of PS II subunit 6
Lhcb7	Light harvesting complex of PS II subunit 7
L	Litre
LB	Luria-Bertani
µg	Microgram
µL	Microlitre
µmole	Micromole
mL	Millilitre
MMt	Million Metric Tons
MCSM	Modified Cross-Streak Method
MF	Molecular Function
H_2PO_4	Monobasic Phosphoric Acid
N	Nitrogen
NRE	N Remobilization Efficiency
NTE	N Transport Efficiency
NUpE	N Uptake Efficiency
NUtE	N Utilization (Assimilation) Efficiency
N^+PGPR^+	N-Appropriate With Microbes
N^+PGPR^-	N-Appropriate Without Microbes
N^-PGPR^+	N-Deficit With Microbes
N^-PGPR^-	N-Deficit Without Microbes
NR	Nitrate Reductase
NUE	Nitrogen Use Efficiency
NB	Nutrient Broth
NF	Nutrient Formulation
OP	Organic Phosphate
PSI	Phosphate Solubilisation Index
PSB	Phosphate Solubilizing Bacteria
PSMs	Phosphate Solubilizing Microorganisms
P	Phosphorus
PPM	Phytase Production Media
PSM	Phytate Screening Media
PKA	Pikovskaya Agar
PKB	Pikovskaya Broth
PGPR	Plant Growth Promoting Rhizobacteria
PGP	Plant Growth Promotion
PYR/PYL	Polyketide cyclase/dehydrase and lipid transport superfamily protein
K	Potassium

PCA	Principal Component Analysis
Lhca5	PSI light harvesting complex protein 5
Lhca2	PSI type II chlorophyll a/b- binding protein
Lhca3	PSI type III chlorophyll a/b- binding protein
Lhcb1	PSII Light harvesting complex protein 1
Lhcb2	PSII Light harvesting complex protein 2
NO*x*	Reactive N Gases
RSM	Response Surface Methodology
RT	Room Temperature
RPM	Rotation Per Minute
SAUR	SAUR-like auxin-responsive protein family
SRA	Sequence Read Archive
SnRK2	Sucrose nonfermenting 1(SNF1)-related protein kinase
SOD	Superoxide Dismutase
TF	Transcription Factor
AUX/IAA	transcriptional regulator family protein
AUX1	Transmembrane amino acid transporter family protein
TCP	Tricalcium Phosphate
TCA	Tri-Chloroacetic Acid
VOCs	Volatile Organic Compounds
Zn	Zinc
ZC	Zinc Carbonate
ZP	Zinc Phosphate
ZSI	Zinc Solubilisation Index

Appendix B

PUBLICATIONS AND ABSTRACTS

(A) Publications

Deepika Mazumdar, Shyama Prasad Saha, Shilpi Ghosh (2019). Isolation, screening and application of a potent PGPR for enhancing growth of Chickpea as affected by nitrogen level. International Journal of Vegetable Science 26 (4), 333-350.

Deepika Mazumdar, Shyama Prasad Saha, Shilpi Ghosh (2018) *Klebsiella pneumoniae* RS26 as a potent PGPR isolated from chickpea (*Cicer arietinum*) rhizosphere. The Pharma Innovation Journal 7(11), 56-62.

Moushree Pal Roy, **Deepika Mazumdar,** Subhabrata Dutta, Shyama Prasad Saha, Shilpi Ghosh (2016). Cloning and expression of Phytase appA gene from *Shigella* sp. CD2 in *Pichia pastoris* and comparison of properties with recombinant enzyme expressed in *E. coli.* PLoS ONE.

(B) Abstracts

Deepika Mazumdar and Shilpi Ghosh (2015). Adverse effect of Nitrogen fertilizers in the Environment and implications of Plant Growth Promoting Rhizobacteria in curtailing their use. International Symposium on Biodiversity, Agriculture, Environment and Forestry, held on 11-12th December, 2015, organized by the Association for the Advancement of Biodiversity Science (Oral).

Deepika Mazumdar, Shyama Prasad Saha and Shilpi Ghosh (2019). Application of two potent PGPR on mustard plant under differential Nitrogen input. International seminar on Current Avenues in Microbial and plant Sciences, held on 23-24-25th February, 2019, organized by Department of Botany, University of Gourbanga, West Bengal (Oral).

Deepika Mazumdar, Shyama Prasad Saha and Shilpi Ghosh (2019). Optimization of potent PGPR dosage using Response Surface Methodology and their application on *Brassica campestris* plant under differential Nitrogen input. National seminar on Vistas in Life Science Research, held on 29th March, 2019, organized by Departments of Biotechnology, Microbiology, Tea science and Bioinformatics, University of North Bengal, West Bengal (Oral)

Deepika Mazumdar, Shilpi Ghosh (2020). Response Surface Methodology: An innovative tool to optimize potent PGPR dosage and minimize the use of inorganic fertilizer on *Brassica campestris* L. National seminar on Recent Advances in Translational Research, held on 29th February, 2020, organized by Department of Biotechnology, University of North Bengal, West Bengal (Oral).

Deepika Mazumdar and Shilpi Ghosh (2014). Screening of rhizospheric bacteria for multiple PGPR activities and their application on the growth of chickpea (*Cicer arientinum*) under different nitrogen conditions. National seminar on Applied Microbiology: Microbial World, held on 14th March, 2014, organized by Dept. of Microbiology, University of North Bengal (Poster).

Deepika Mazumdar and Shilpi Ghosh (2015). Role of Plant Growth Promoting Rhizobacteria supplementation on the growth of chickpea (*Cicer arientinum*): An attempt to minimize nitrogen input in the environment. 22nd West Bengal State Science & Technology Congress, held on 28th February and 1st March, 2015, organized by Department of Science and Technology (Govt. of West Bengal), West Bengal State Council of Science and Technology and University of North Bengal (Poster).

Deepika Mazumdar, Shyama Prasad Saha and Shilpi Ghosh (2020). Immobilization of xylanase obtained from *Bacillus* sp xym 12 in alginate beads for improving its thermostability and reusability. National seminar 'Microbial World 2020' on Applied Microbiology, held on 27th February, 2020, organized by Department of Microbiology, University of North Bengal, West Bengal (Oral).

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Isolation, screening and application of a potent PGPR for enhancing growth of Chickpea as affected by nitrogen level

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ABSTRACT

Chickpea (*Cicer arietinum* L.) can obtain much of its N requirement through nitrogen fixation, but plant productivity also relies on N fertilization. Nitrogen capture from fertilizers by the plant is highly inefficient and can have a negative impact on the environment. Plant growth promoting rhizobacteria (PGPR) can improve efficiency of N fertilizer use and support optimum plant growth under low N input to soil. Chickpea plants were grown in pots and treated with low and optimum N input supplemented with PGPR. Bacteria isolated from chickpea rhizosphere exhibited potential growth promoting production of ammonia, indole acetic acid and phytase and solubilization of inorganic phosphate and zinc. A PGPR strain, identified as *Cedecea davisae* RS3, was selected by principal component analysis and its effect on plant growth promotion evaluated in chickpea plants under N-appropriate (N^+), N-deficit with RS3 (N^-RS3) and N-appropriate with RS3 (N^+RS3) treatments. Overall performance of chickpea plants grown under N^-RS3 was better than that of N^+ and N^+RS3 plants. The improved performance under N-RS3 could be related to enhancement of root nodulation and nitrogenase activity. The PGPR with N-RS3 might be used to obtain sustainable production of chickpea under reduced N.

KEYWORDS

Cedecea davisae RS3; *Cicer arietinum*; glutamine synthetase; principal component analysis; rhizosphere

Chickpea (*Cicer arietinum* L.) even though a legume, requires N fertilization, especially for root and shoot growth (Demirbas et al., 2018). In chickpea, N is mainly transported through xylem and phloem as amino acids, ureides, allantoin, allantoic acid and amides. Enhanced level of these N compounds in plants is affected by N-fertilization (Vessey and Layzell, 1987).

In agricultural soils, mineral N is generally supplied in fertilizers as ammonium (NH_4^+) and nitrate (NO_3^-). The amount of N-fertilizers added to soil worldwide annually is predicted to increase by 2050 (Tilman, 1999). Together with crop breeding, application of chemical N-fertilizers has increased global

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food production. However, there is increasing concern regarding sustainability of technology to provide long-term food security to a growing population (Hera, 1995; Xu et al., 2012).

Nitrogen capture from fertilizers by crops is relatively inefficient, with 30–65% of applied N being utilized. Excess nitrogenous compounds released from agriculture threaten air, water and soil qualities. Increased N leaching into sub-surface water, release of atmospheric nitrous oxide and reactive nitrogen gases (NO*x*, NH_3) into the troposphere, accelerate eutrophication of waterways and acidify soil (Xu et al., 2012). The NO_3^- accumulated in harvested crops is considered a source of potential danger to human health. The challenge is to continue increasing agricultural productivity in a way that reduces harmful effect of N-fertilizers.

Plant growth promoting rhizobacteria (PGPR) affect plant growth directly due to their ability to supply nitrogen, phosphorous, potassium and other essential minerals to plants, or indirectly interfering with pathogens attacking plants (Kloepper and Schroth, 1981) and can be utilized as an alternative to chemical fertilizers (Aeron et al., 2011; Ribeiro and Cardoso, 2012).

The study was undertaken to screen indigenous PGPR of chickpea rhizosphere for plant growth promoting attributes and evaluate effects of providing a PGPR, *Cedecea davisae* RS3, to the nutrient media under low and optimum N treatment regime, on growth of chickpea plants.

Material and methods

Rhizosphere soil of chickpea plants growing in field at 26°42′ 30.88” N longitude and 88°20′ 52.97” E latitude was collected during December 2015. In total, 1 g of soil was suspended in 10 mL of standard 0.85% saline and vortexed for 10 min to obtain a uniform suspension. Serial dilutions were made by sequentially adding 1 mL to form 10^{-1} through10^{-7} dilutions. The serially diluted suspensions were spread plated on nutrient agar and incubated at 37°C for 24 h. Isolated colonies with distinct colony morphology were selected. They were streaked on agar to obtain pure cultures and stored in glycerol at −20°C. All isolated strains were tested for plant growth promoting attributes.

Bacterial isolates were grown in Asbhy’s N-free liquid medium containing ($g.L^{-1}$) mannitol,15; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.2; $CaCl_2$, 0.2; $FeCl_3$, 0.05, and 10 µL of Na_2MoO_4 (10% w/v) at 37°C for 24 h.The culture was streaked on Asbhy’s N-free agar plates and incubated at 37°C for 24 h. Production of ammonia was quantified by the method of Goswami et al. (2014). Bacterial cultures grown in Asbhy’s N-free liquid medium were centrifuged at 3000 × g for 10 min and 0.2 mL culture supernatant mixed with 1 mL Nessler’s reagent and total volume made to 8.5 mL by adding ammonia-free distilled water. Development of brown to yellow color is indicative of ammonia production which was estimated spectrophotometrically at 450 nm using a standard curve prepared with 0.1–10 µmol ammonium sulfate.

Growth capacity of isolates in N-free medium was detected by the method of Ribeiro and Cardoso (2012). Isolates were grown in screw-cap tubes containing 10 mL Jacob's nitrogen-free (JNF) semi-solid culture media without N and incubated at 37°C for 7 days. Pellicle-forming isolates were streaked on JNF agar plates supplemented with 20 $mg.mL^{-1}$ yeast extract. Isolates growing on JNF agar plates were inoculated into JNF semi-solid media.

Production of indole acetic acid (IAA) was determined by the method of Patten and Glick (2002). Bacterial cultures were grown in Luria broth (LB) supplemented with tryptophan (1 $mg.mL^{-1}$) at 37°C with shaking at 120 rpm for 2 days followed by centrifugation at 3000 × g for 10 min. In total, 1 mL of supernatant was combined with 2 mL of Salkowski's reagent (150 mL of 95–98% H_2SO_4, 7.5 mL of 0.5 M $FeCl_3.6H_2O$ and 250 mL distilled water) and incubated for 30 min at 25°C. Development of pink color indicated IAA production and its optical density was recorded at 530 nm. Concentration of IAA was estimated using a standard curve with 1–20 $\mu g.mL^{-1}$ IAA.

Phosphate solubilizing ability was determined by the method of Katznelson and Bose (1959). Bacterial cultures grown for 24 h were spot inoculated on Pikovskaya agar plate (PA) containing dicalcium phosphate (DCP) or tricalcium phosphate (TCP) as insoluble inorganic phosphate sources. Plates were incubated at 37°C for 7 days and observed for appearance of a clear zone around colonies. Phosphate solubilization index was calculated following Vazquez et al. (2000). Solubilized phosphate content was estimated by the vanadomolybdophosphoric acid method (Barton, 1948). Concentration of soluble phosphate was estimated using a standard curve of KH_2PO_4.

Organic phosphate solubilization was determined by the ability of bacterial isolates to hydrolyze phytate by producing the enzyme phytase. Isolated organisms were grown in phytate screening media (PSM) containing (%): glucose, 2; KCl, 0.05; $CaCl_2$, 0.2; NH_4NO_3, 0.5; $MgSO_4$, 0.05; $MnSO_4$, 0.001; $FeSO_4$, 0.001; sodium phytate, 0.2; and agar, 1.5 (at pH 7) at 37°C for 2–3 days. Formation of a clear zone around the colony indicates phytase production. Phytase-positive strains were analyzed for phytase activity as described by Pal Roy et al. (2016). Amount of inorganic phosphate (Pi) released from phytate was determined using a Pi standard curve. One unit (U) of phytase activity represents 1 μmol of Pi $released.min^{-1}$.

Bacterial cultures grown for 24 h were spot inoculated on zinc solubilization medium containing 1% $ZnPO_4$ or $ZnCO_3$ as the zinc source (Kumar et al., 2014) and incubated at 37°C for 5 days. Zinc solubilization index was calculated using the equation of Vazquez et al. (2000).

A PGPR strain with almost all the PGP attributes of ammonia production, organic and inorganic phosphate solubilization, IAA production and zinc

solubilization, making it potent, was selected with principal component analysis (PCA) using SPSS (ver. 16, SPSS Inc., Chicago, IL). The bacterial strain RS3 was selected for further study. The bacterial strain was grown in nutrient broth and O.D. values recorded at 610 nm at an interval of 30 min to determine growth patterns.

Identification of PGPR bacterial strain RS3 was by sequence analysis of the 16S rRNA gene. Genomic DNA was isolated using HiPura genomic DNA isolation kit (HiMedia, Mumbai, India) following manufacturer instructions. A 25 µL PCR reaction mix containing 5× GoTaq Flexi buffer, 5.0 µL; 25 mM $MgCl_2$, 2 µL; 10 pmoles of each universal primer (forward primer 27F 5′-AGAGTTTGATCCTGG CTCAG-3′and reverse primer 1492R 5′ TACGGTTACCTTGTTACGACTT-3′); 10 mM dNTPs, 1 µL; genomic DNA, 100 ng and 2.5 U of Taq DNA polymerase and PCR was carried out for 30 cycles. The PCR product was cloned in the pGEM-T vector system (Promega, Madison, WI) following manufacturer's instruction and sequenced. The phylogenetic relationship of isolate RS3 was determined by comparing its 16SrDNA sequence with closely related neighbor sequences retrieved from the GenBank database of the National Center for Biotechnology Information via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Astchul et al., 1990).

A pot experiment was carried out in a greenhouse maintained at 25 ± 2°C and 70–80% relative humidity. Chickpea Seeds were disinfected with 1% sodium hypochlorite for 10 min followed by a thorough washing with sterile distilled water and seed were placed in an incubator at 25°C for 2–3 days in the dark. Surface soil (~15 cm depth, pH 6–6.5), collected from a non-fertilized field site, was dried, sieved, autoclaved for 1 h and placed into earthen pots (15 cm dia) until 3/4 filled. In total, 100mL of RS3 culture (10^7 cfu.mL^{-1}) was centrifuged at 5000 × g at 4°C for 10 min and the cell pellet, suspended in 100 mM phosphate buffer (pH 7), used as inoculum for the pot experiment.

Six germinated seeds were transferred to each pot and pots were grouped into 3 treatments with 9 pots in each group. The groups were: N-appropriate (N^+), N-deficit with RS3 (N^-RS3), and N-appropriate with RS3 (N^+RS3). The N^+ plants were irrigated with nutrient solution containing $(NH_4)_2SO_4$, 5 mM; NaH_2PO_4, 0.3 mM; K_2SO_4,0.5 mM; $CaCl_2$,1 mM; $MgSO_4$,1.6 mM; Fe-EDTA, 0.05 mM; $Na_2MoO_4.2H_2O$, 0.06 µM; H_3BO_3, 0.015 mM; $MnCl_2$, 0.008 mM; $ZnSO_4$, 0.12 µM, and $FeCl_3$, 0.029 mM. For N-appropriate (N^+) treatment, the concentration of N in the nutrient solution was 5 mM $(NH_4)_2SO_4$ (Mollar et al., 2011). For the N^+RS3 treatment, nutrient solution with appropriate N was used for N^+ plants supplemented with RS3 (10^7cfu·mL^{-1}). For N-deficit with RS3 (N^-RS3), the level of N was reduced to 0.5 mM but supplemented with RS3. Treatment formulations were applied to plants twice weekly. Seedlings were harvested at 7, 15 or 30 days after treatment (DAT), frozen in liquid nitrogen and stored at −80°C until used. Fresh weight, root length and shoot length were

determined on seedlings immediately after harvest. For determination of dry weight, plant samples were dried in a convection oven at 80°C for 48 h and weighed.

Chickpea leaflet tissue was extracted in 80% chilled acetone followed by centrifugation at 8000 × g for 10 min at 4°C. Absorbance of the clarified supernatant was recorded at 663 nm and 645 nm. Chlorophyll content was determined using equations of Arnon (1949). Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Carbohydrate content was determined by the anthrone method using glucose as the standard (Yemm and Willis, 1954).

Root and shoot tissues were homogenized in extraction buffer containing 100 mM Tris Cl (pH 7), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15% glycerol (5 mL·g^{-1} fresh weight). Filtered extract was centrifuged at 8000 × g for 15 min at 4°C. Glutamine synthetase (GS, EC 6.3.1.2) activity in supernatant was determined by transferase reaction (Singh and Ghosh, 2013). The amount of γ-glutamyl hydroxamate produced was determined from a reference curve prepared with 0–2 µmol γ-glutamyl hydroxamate.

Data, means of triplicates, were subjected to one- and two-way ANOVA followed by Tukey's *post hoc* test in SPSS (ver. 16, SPSS Inc., Chicago, IL). If interaction were significant, it was used to explain the data. If the interaction was not significant, means were separated with Tukey's test.

Results and discussion

Management of agricultural soil is fundamental to ensure sustainable agriculture. However, application of large amount of inorganic fertilizers can lead to degradation of soil. The effect of N-fertilizer to crops has been correlated with energy and environmental costs (Riccardo et al., 2014). Nitrogen pollution poses a greater challenge than carbon because of the effect of N through a reactive N cascade of many chemical forms (Xu et al., 2012).

Use of PGPRs is increasing in agriculture because it offers an alternative to chemical fertilizers (Cummings, 2009). PGPRs support plant growth promotion via several direct and indirect mechanisms. They fix atmospheric N into plant utilizable forms. The siderophores produced sequester iron from soil to form complexes taken up by plants as a source of iron (Beneduzi et al., 2012; Crowley and Kraemer, 2007). They have metabolic pathways for synthesis of auxins, cytokinin and gibberellins (Ahemad and Kibret, 2014). They solubilize phosphorus and zinc making them available to plants, and produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase that hydrolyze ACC, the immediate precursor of ethylene thereby lowering the level of ethylene (Patten and Glick, 2002). The ability of PGPR to act against phytopathogens as biocontrol agents take place through siderophore production, antibiotic production, production of hydrogen cyanide, induced systemic resistance,

and production of chitinase enzymes (Glick et al., 2007). The PGPRs isolated from rhizosphere of chickpea were screened for PGP traits and the effect of a potent PGPR, *Cedecea davisae* RS3 supplementation to the growth medium evaluated on performance of chickpea plants grown under differential N input: N-appropriate, N-deficit with RS3 (N^{-}RS3) and N-appropriate with RS3 (N^{+}RS3). Plants grown under N^{-}RS3 exhibited either comparable, or greater levels of chlorophyll a/b, carbohydrate, protein and glutamine synthetase activity, compared to plants grown under N-appropriate (N^{+}) treatment, and had greater root and shoot lengths and biomass.

Analysis of chickpea rhizosphere soil sample produced approximately10^{8}-cfu·$g^{-1}$of soil on nutrient agar. Of 82 morphologically distinct bacterial isolates, 27 exhibiting the ability to fix atmospheric N were screened for other PGP traits. Among the N fixing isolates, 12 produced IAA, 22 solubilized DCP, 6 solubilized TCP, 14 and 16 isolates produced zones of solubilization in media with zinc phosphate and zinc carbonate, respectively. Bacterial strains RS3, RS9, RS10, RS23, RS26, RS31, RS46, RS48, RS49 and RS51 (Table 1), exhibited at least 4 PGP traits, were selected for quantitative screening. Some of the PGPRs exhibited multiple PGP traits *in vitro*. Multiple modes of action have been reported to be the reasons for plant growth promotion and disease suppressing ability of PGPRs (Bashan and de-Bashan, 2010; Sayed et al., 2014). Many PGPRs, belonging to different bacterial classes and genera, with multifunctional traits, can have wide application (Rodríguez-Díaz et al., 2008) likely including in chickpea.

Nitrogen uptake through symbiotic N fixation, or non-associative uptake, contributes to N content of crops (Adesemoye and Kloepper, 2009). All the isolates were able to grow in N-free Asbhy's nitrogen media indicating their ability to fix N to ammonia (Figure 1a). Isolates RS3, RS23, RS26 and RS51 produced more than 6 μg·mL^{-1} of ammonia with higher production by RS3 and RS26. Isolates RS3 and RS26 formed pellicles in the JNFb semi-solid N-free medium and were categorized as free-living N fixers. The IAA

Table 1.: Plant growth promoting attributes by isolates.

		Inorganic			Solubilization		
Isolate	Nitrogen fixation	DCP	TCP	Organic	$ZnCO_3$	$ZnPO_4$	IAA production
RS3	+[a]	+	+	+	+	+	+
RS7	+	+	+	-	-	-	+
RS9	+	+	-	-	+	+	+
RS10	+	+	-	-	-	+	+
RS23	+	+	-	-	+	-	+
RS26	+	+	-	+	+	+	+
RS31	+	+	-	-	-	+	+
RS46	+	+	-	-	+	-	+
RS48	+	-	+	-	+	-	+
RS49	+	+	-	-	+	-	+
RS51	+	+	-	-	-	+	+

[a]+ = positive response; – = negative response.

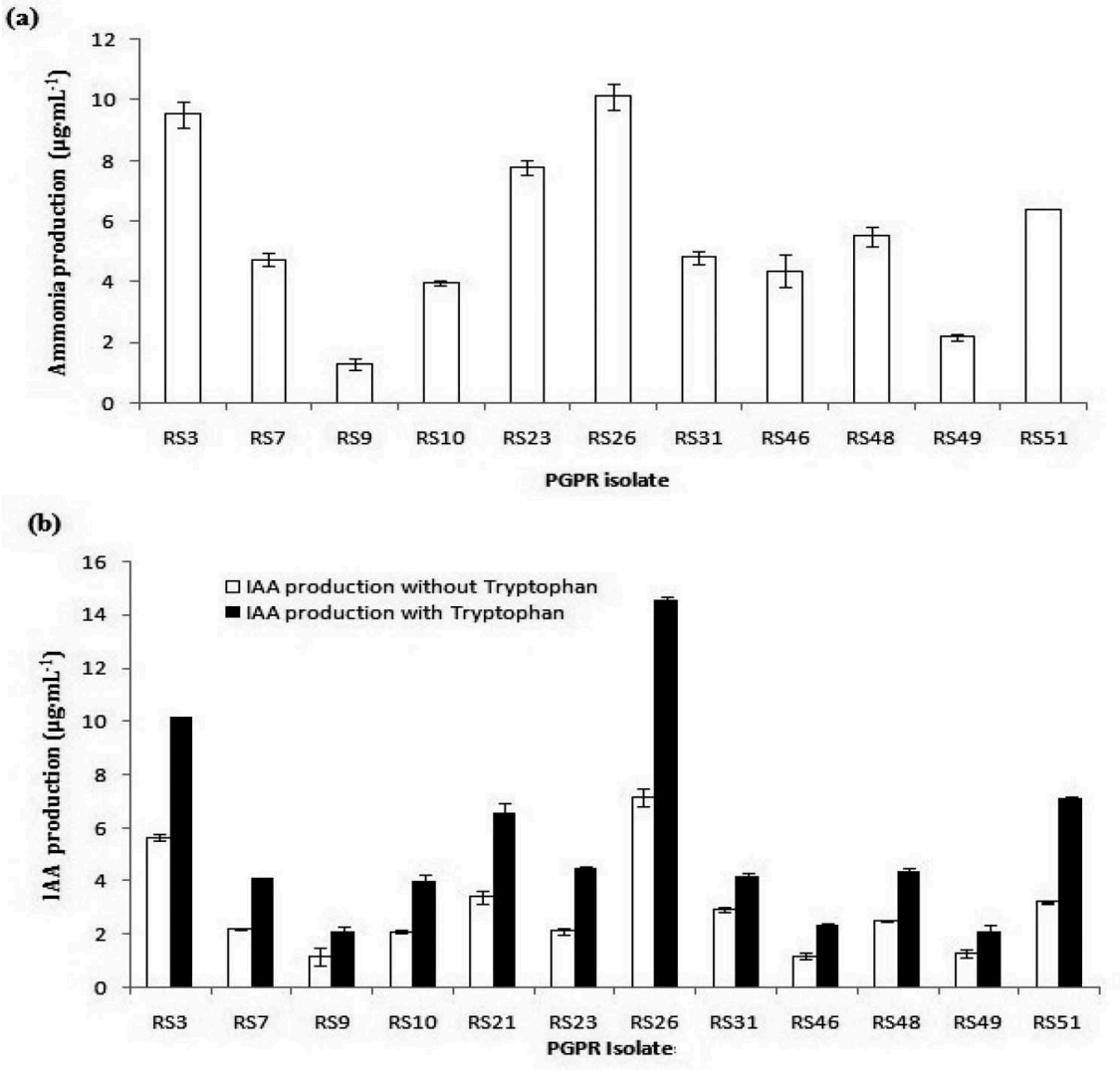

Figure 1. (a). Quantitative estimation of ammonia production by PGPR isolates in Asbhy's N-free liquid medium at 24 h of growth, (b) Quantitative estimation of IAA, produced by selected isolates in a medium with and without tryptophan (1 mg·mL^{-1}). Data are triplicate mean ± SD.

synthesized endogenously by plants is associated with apical dominance, tropism, shoot elongation, induction of cambial cell division and root initiation (Olanrewaju et al., 2017; Ribeiro and Cardoso, 2012); however, its hormonal effects on plants can occur by exogenous application. All chickpea rhizosphere bacteria were able to synthesize IAA in absence, or presence, of tryptophan with relatively greater production in presence of tryptophan (Figure 1b). Bacterial strains RS3 and RS26 produced greater quantity of IAA and production levels were lower in absence of than in presence of tryptophan. Rhizobacteria mainly use tryptophan as a precursor for IAA synthesis, however, tryptophan-independent pathways have been described (Ribeiro and Cardoso, 2012), which explains production of IAA by chickpea rhizobacteria in absence of tryptophan. Up to 80% of IAA synthesizing bacteria colonize root surfaces and act in conjunction with endogenous IAA to stimulate the root system to increase size and number of adventitious

roots, enabling them to take up more nutrient from soil for plant growth (Patten and Glick, 2002). Phytohormones produced by rhizobacteria are more effective by virtue of their continuous and slow release (Mohite, 2013).

Phosphate is an essential nutrient for plant growth having roles in photosynthesis, respiration, macromolecules biosynthesis, and energy transfer (Huang et al., 2008). In soil, organic and inorganic phosphates are strongly complexed and insoluble and poorly available to plants. Phosphate solubilization by rhizobacteria is important because plants can absorb and assimilate only inorganic phosphate. The major phosphate solubilization mechanisms of PGPRs include release of the mineral dissolving compounds organic acid anions, protons, and extracellular secretion of phosphate hydrolyzing enzymes (Pal Roy et al., 2017; Richardson et al., 2011). Chickpea rhizophere bacteria had the ability to hydrolyze organic and inorganic phosphates. Isolates RS3, RS10, RS23, RS26, RS46 and RS49 had a phosphate solubilization index (PSI) of≥2 (Figure 2a). Most selected isolates except RS46, formed zones of solubilization on DCP containing Pikovskaya's agar, whereas RS3 and RS46 formed solubilization zone on TCP, indicating inorganic phosphates solubilization (Figure 2b). The relatively greater PSI of RS3, RS23 and RS49 was significantly correlated with their higher liberation of Pi (Figure 2c). The release of Pi was associated with lowered medium pH from neutral for RS3, RS23 and RS49, which could be due to secretion of citric, propionic, gluconic, succinic, oxalic, acetic, formic and lactic acids which are most common in phosphate solubilization (Chen et al., 2006; Wei et al., 2018).

Organic phosphate in soil is mainly present as phytic acid and salt phytate which is unavailable to plants and it becomes necessary to determine the rhizospheric bacteria that produce phytase (Pal Roy et al., 2017). Isolates RS3, RS23, RS26 and RS49 produced extracellular phytase with substantial activity in the pH range 4.5–7.5 indicating their ability to hydrolyze phytate in soil with similar pH values. Strain RS26 produced the highest phytase activity at pH 6.5 at 48 h of incubation (1.9 μmole.mL^{-1}·min^{-1}). Strain RS3 had the highest phytase activity (1.2 μmole.mL^{-1}·min^{-1}) at pH 4.5 at 24 h incubation with a decrease at 48 h incubation. Strains RS23 and RS 49 produced 1.1 μmole.mL^{-1}·min^{-1}of phytase at pH 7.5 at 48 h incubation and 1.2 μmole.mL^{-1}·min^{-1} at pH 6.5 at 24 h of incubation. Most bacterial cultures produced phytase by 48 h of incubation.

Other than phosphorus, zinc is an essential element required for optimum plant growth. It is a micronutrient whose deficiency in plants leads to reduced carbohydrates, auxins, nucleotides, cytochromes, and chlorophyll synthesis; reduced integrity of membrane, and increased susceptibility to high temperature (Singh et al., 2005). Excessive use of Zn impairs iron and copper absorption in humans (Singh et al., 2005). Bacterial isolates capable of solubilizing Zn in soil are considered as potential alternates to Zn fertilization. The PGPR isolates RS3, RS9, RS23, RS26, RS46, RS48 and RS49 solubilized $ZnCO_3$, whereas RS10, RS31

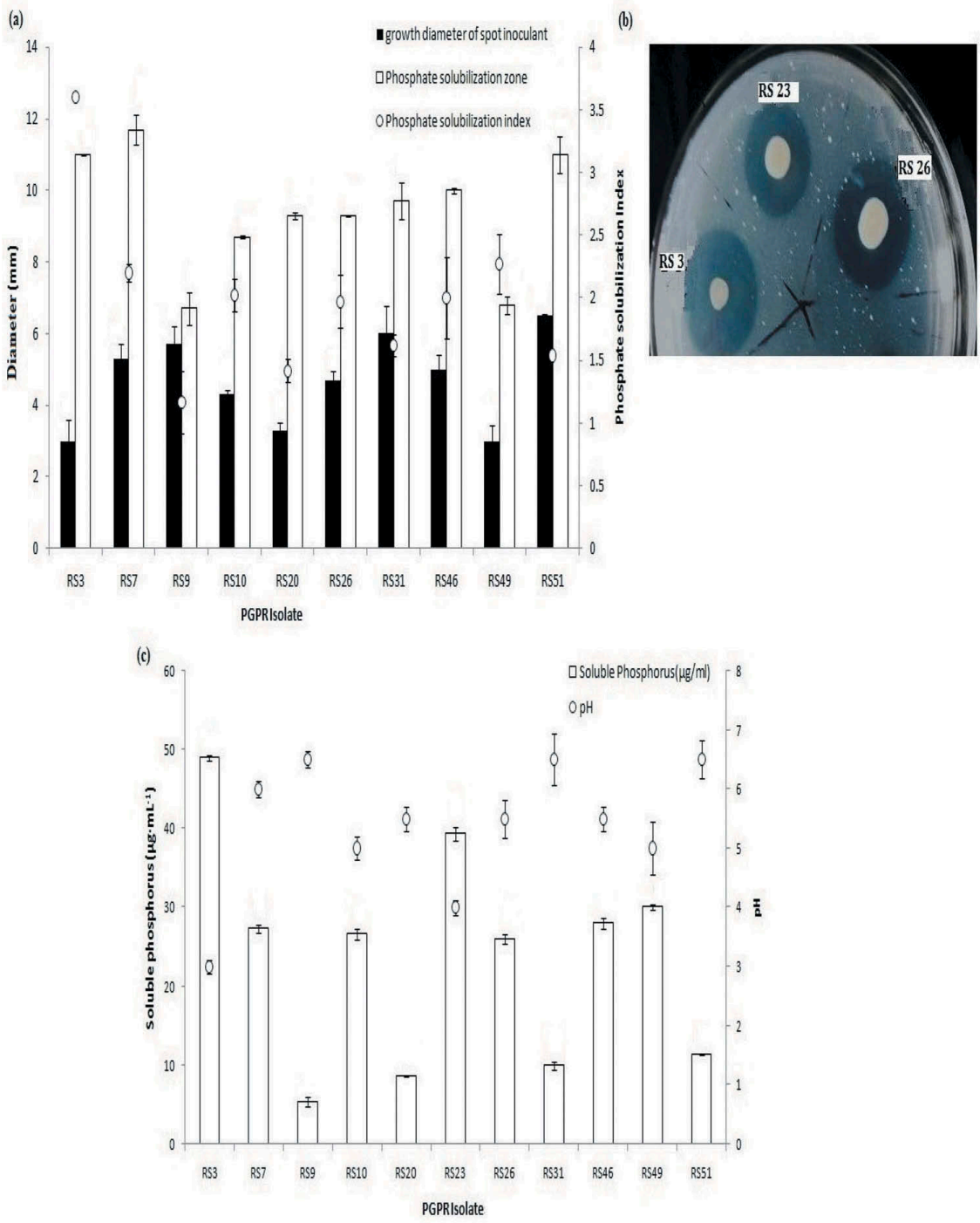

Figure 2. (a) Phosphate solubilization index (PSI) created by phosphate solubilizers on Pikovskaya's agar medium, on Y-axis diameter (mm) refers to colony growth and phosphate solubilization zone, (b) Clear zone indicates solubilization of dicalcium phosphate in medium plate by PGPR strains RS3, RS10 and RS26, and (c) Quantitative estimation of liberated inorganic phosphate obtained during solubilization of dicalcium phosphate by selected phosphate solubilizers. Data are triplicates of mean ± SD.

and RS51 solubilized $ZnPO_4$. Isolates RS9, RS23 and RS26 have the maximum zinc solubilization index of about 2 (Figure 3).

The PGPR isolate used for the pot experiment was selected by principal component analysis (PCA) using SPSS. A combined approach for selection of factors based on Eigen values, scree plot and variance explained criterion of

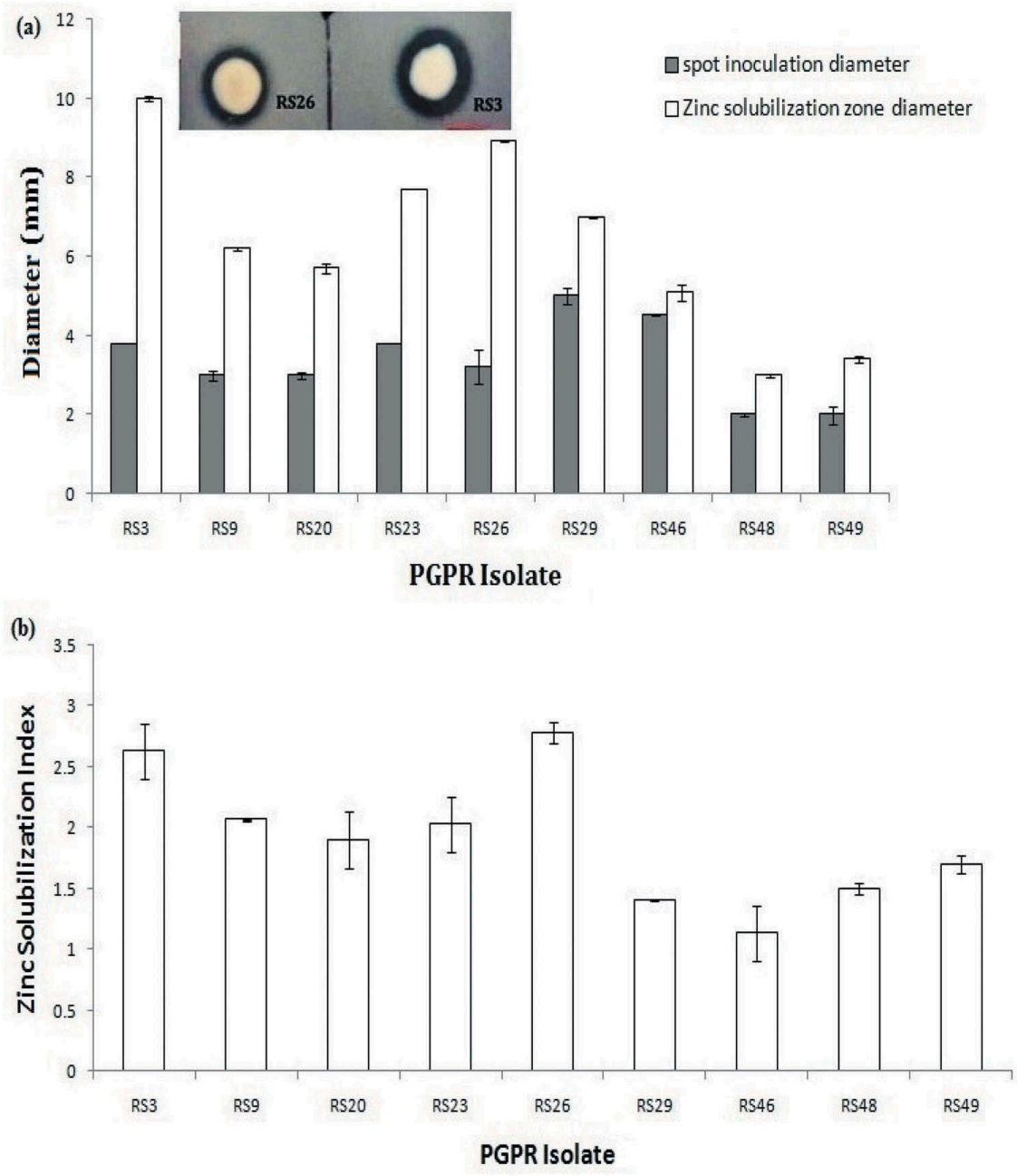

Figure 3. (a) Diameter refers to colony growth and zinc solubilization zone by PGPR isolates, embedded figure is solubilization of zinc carbonate by PGPR isolates RS26 and RS3. In the embedded figure, the halo around the growth of RS26 and RS3 represent the solubilization of insoluble zinc carbonate containing media. (b) Zinc solubilization index created by PGPR zinc solubilizers. Data are triplicates of mean ± SD.

60%, considered 2 principal components (PCs), PC1 and PC2 which explain 32.7 and 30.1% variability, respectively. Major contributory factors to PC1 were ammonia production (0.857), IAA production without tryptophan (0.878), IAA production with tryptophan (0.874), phosphate solubilization (0.633). The microbial strains RS3 and RS26 contributed to PC1 with correlation coefficients of 0.607 and 0.602, respectively. The second principal component PC2 represented phosphate solubilization zone diameter (0.875)

and final pH of phosphate solubilizing media (0.846). The principal component loading plot indicated microbial strains RS3 and RS26 had significant, positive, contributions to most PGP attributes of PC1as reflected by formation of a tight cluster. The PCA indicated RS3 and RS26 as potent PGPRs, and RS3 was selected for the pot trial experiment. Phylogenetic analysis based on 16S rRNA gene sequence identified isolate RS3 as *Cedacea davisae* and was designated *C. davisae* RS3 (GenBank accession number KX101223).

Protein contents of root and shoot increased with age in all groups, with N^-RS3 and N^+RS3 having significantly greater levels compared to N^+ plants (Figure 4a). The higher level of chlorophyll b in N^-RS3 plant shoots was correlated with their greater carbohydrate content compared to that of N^+ and N^+RS3 plants (Figure 4b). Protein synthesis requires synergy of carbon and nitrogen metabolism. Higher chlorophyll b, carbohydrate and ammonia assimilation (as reflected by GS activity) in shoots of N^-RS3 and N^+RS3 plants could account for higher protein content.

Fresh weight of roots of N^-RS3 and N^+RS3 plants were greater than that of N^+ (Figure 5). Shoot fresh weight of N^-RS3 plants was almost 2 × greater than for N^+ and N^+RS3 plants. Although root length was almost similar in all treatment groups until day 7, it increased in N^-RS3 and N^+RS3 plants at 15 and 30 days.

Chlorophyll a level gradually increased from day 7 to day 30 of growth, however, it was not different between the three treatment groups (Figure 6a). Chlorophyll b level of N^-RS3 and N^+RS3 plants was greater than that of N^+ plants throughout the treatment duration. Chlorophyll b level of N^-RS3 plants was 1.5 × greater than N^+RS3 at 30 days of treatment. Chlorophyll b is an antenna chlorophyll and its binding to the antenna protein is crucial for correct assembly of the thylakoid membrane (Hoober et al., 2007). Chlorophyll b has a major impact on lateral mobility and diffusion of membrane molecules, light harvesting and thermal energy dissipation processes, electron transport, and repair processes in grana (Hoober et al., 2007). Significant increases in yields of different crop plants occurs following PGPR application under natural niches and controlled environments (Ahemad and Kibret, 2014). Plant growth promotion by PGPRs occur in chickpea (Pal Roy et al., 2017) and other crops (Egamberdiyeva, 2007; Goswami et al., 2014) indicating their broad application as an effective tool for sustainability. Due to existing reluctance worldwide to use foods produced by genetically modified plants, PGPRs may be beneficial as a means of improving plant growth. The wide scale application of PGPRs may reduce dependence on agricultural chemicals. Use of PGPRs is within reach of farmers in developed and developing countries (Gamalero et al., 2009).

In higher plants, ammonia either absorbed from soil, or produced via nitrate reduction and biological nitrogen fixation, is mainly assimilated to glutamine by activity of glutamine synthetase (GS, EC 6.3.1.2) and GS activity can serve as a measure of ammonia uptake and assimilation (Singh and Ghosh, 2013). The

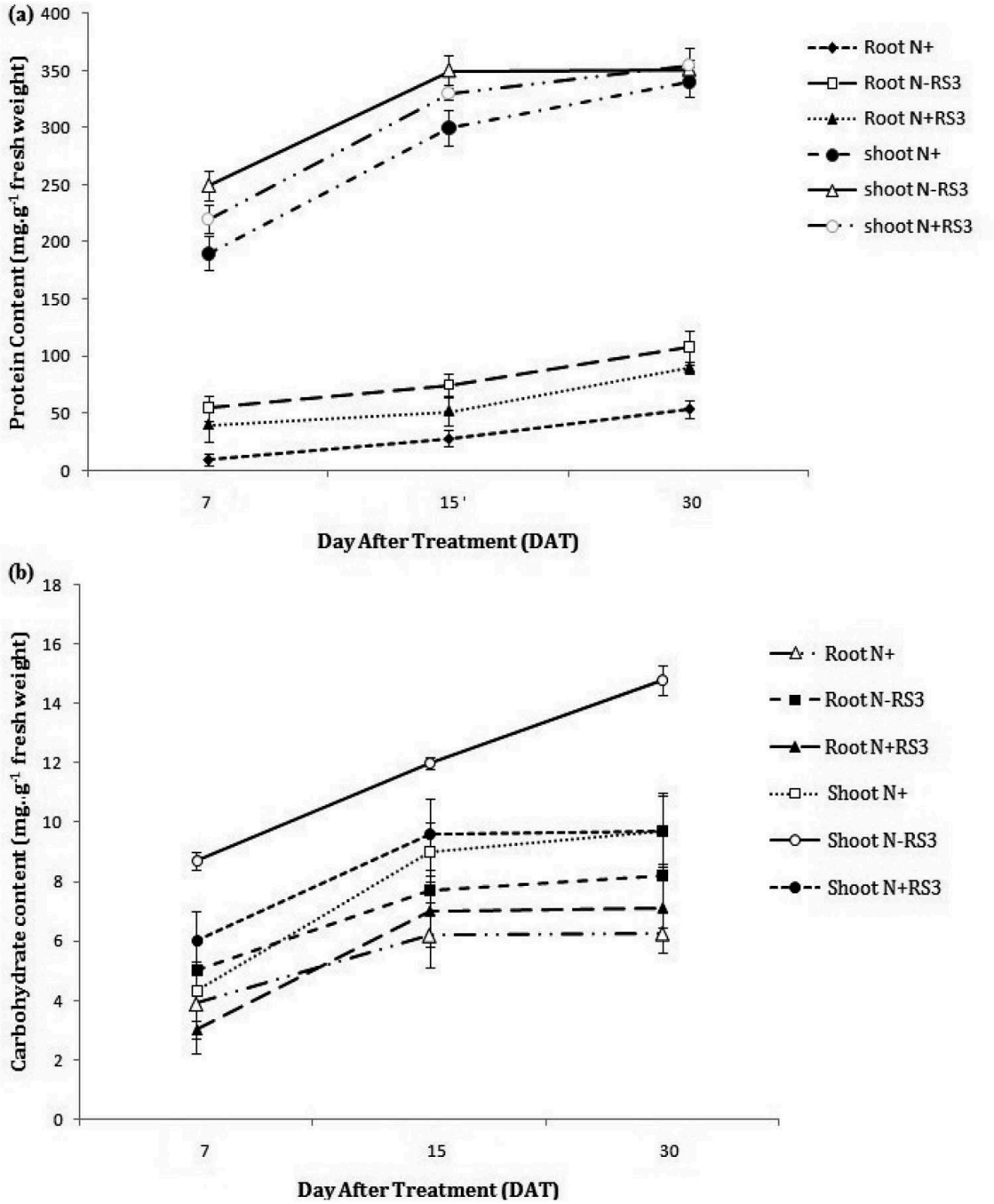

Figure 4. (a). Effect of PGPR treatment on protein content in chickpea, (b) Effect of PGPR treatment on carbohydrate content. Data are triplicate mean ± SD. For each PGPR isolate one-way ANOVA was followed by Tukey's post hoc test. For each biochemical parameter (carbohydrate and protein content of root and shoot) two-way ANOVA was followed by Tukey's post hoc test. A significant interaction effect occurred between treatment condition and treatment time (DAT), for carbohydrate and protein content of root and shoot [treatment condition × treatment time (DAT) $p < .001$ and $R^2 = 1$, Adjusted $R^2 = 0.998$].

GS activity of shoots and roots increased up to 15 days of growth and then declined (Figure 6(b,c)). The enzyme activity responded differently in roots and shoots due to N treatment. The GS activity in shoots of N^+RS3 and N^-RS3 plants was about 3–4 × greater than of N^+ plants; GS activity in roots of N^+ plants was

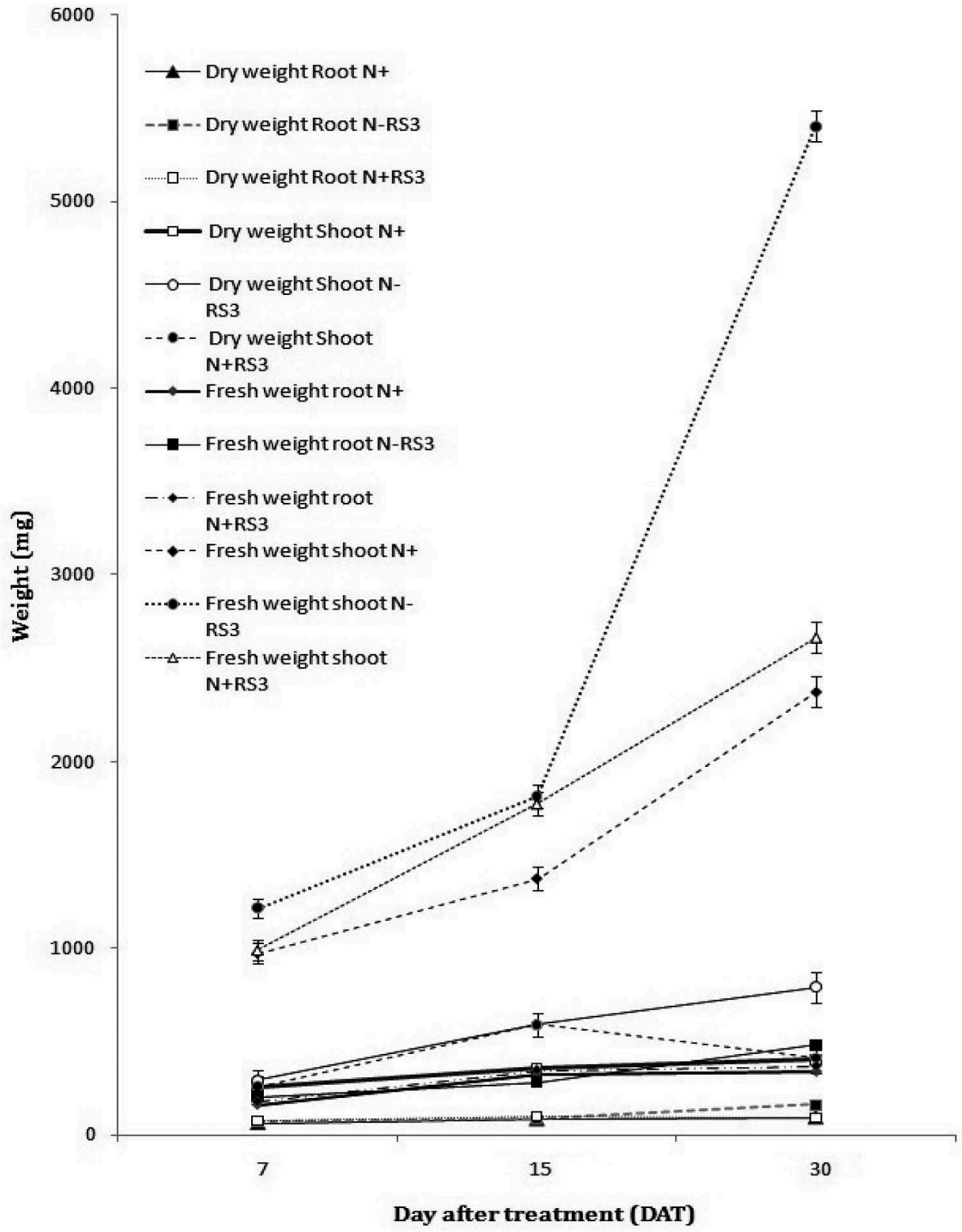

Figure 5. Effect of treatment on shoot and root fresh and dry weights of chickpea plants at 7, 15 and 30 DAT. Data are triplicate mean ± SD. For shoot and root, fresh and dry weights two-way ANOVA was followed by Tukey's post hoc test. A significant interaction occurred between treatment condition and treatment time (DAT), for shoot and root fresh and dry weights [treatment condition × treatment time (DAT) $p < .001$ and $R^2 = 0.997$, Adjusted $R^2 = 0.998$].

greater than that of other 2 groups. The shoot, especially leaves act as sink for N during vegetative stage (Xu et al., 2012), and enhanced GS activity in shoots of N^+RS3 and N^-RS3 plants up to 15 days of growth indicates the role of bacterial inoculum in improving uptake and assimilation of ammonia. Plant uptake of

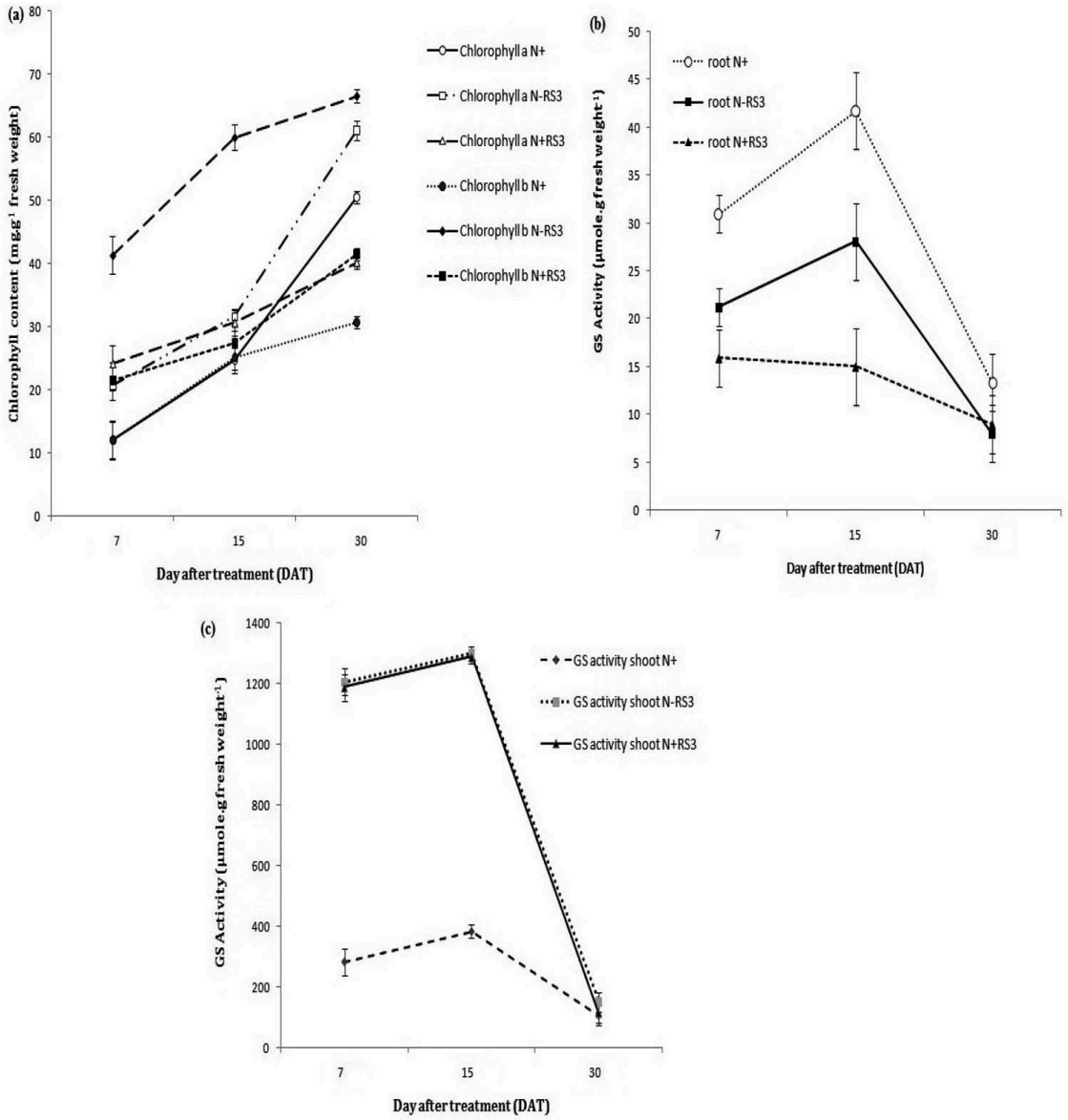

Figure 6. Effect of treatment method on growth related biochemical parameters of chickpea plants at 7, 15 and 30 DAT. (a) Effect of treatment on chlorophyll a and chlorophyll b (mg·g^{-1} fresh weight), (b) Effect of treatment on glutamine synthase (GS) activity of root (µmole·g^{-1}fresh weight), (c) Effect of treatment on glutamine synthase (GS) activity of shoot (µmole·g^{-1}fresh weight). Data are triplicate mean ± SD. For chlorophyll level and GS activity of root and shoot, two-way ANOVA was followed by Tukey's post hoc test. A significant interaction effects occurred between treatment condition and treatment time (DAT), for chlorophyll level and GS activity of root and shoot [treatment condition × treatment time (DAT) $p < .001$ and $R^2 = 0.997$, Adjusted $R^2 = 0.998$].

ammonium produced through N-fixation requires alteration in bacterial N metabolism so that nitrogen is excreted rather than incorporated into microbial biomass. Nitrogen metabolism is significantly altered during rhizobium-legume symbiosis with ammonia-assimilation being effectively shut down in bacterium. Inactivation of ammonium assimilation maybe accomplished via an unknown and probably plant-regulated post translational modification of GS (Bravo and Mora, 1988; Patriarca et al., 2002).

Inoculation of PGPRs, along with inorganic N fertilizer, resulted in increased yields comparable to, or greater than, when conventional quantities of inorganic N is applied. In soybean (*Glycine max* L.), also a legume, root nodulation and nitrogenase activity (key enzyme for nitrogen fixation) was accelerated by low N concentrations (<50 $mg{\cdot}L^{-1}$) and suppressed by high concentrations of nitrogen (>50 $mg{\cdot}L^{-1}$) (Xia et al., 2017). In this study, performance of chickpea was better in low N concentration supplemented with the *C. davisae* RS3.

Improved growth of N^{-}RS3-treated plants indicated PGPRs improved N uptake by plants. The bacterial strain produced IAA that increased root biomass and surface enabling greater uptake of P, Fe, K, Zn and N for enhanced growth of N^{-}RS3-treated plants. It appears *C. davisae* RS3 may be used as a potential bioinoculant agent for optimum plant growth promotion under N-limiting conditions but this needs to be examined under field conditions.

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Klebsiella pneumoniae rs26 as a potent PGPR isolated from chickpea (*Cicer arietinum*) rhizosphere

Deepika Mazumdar, Shyama Prasad Saha and Shilpi Ghosh

Abstract
In this study, chickpea plant (*Cicer arietinum*) rhizobacteria were screened for plant growth promoting traits and among the isolated PGPR, a potent strain RS26 was selected for further studies. The strain was non-pathogenic to human as determined by its inability to produce hemolysin and was identified by morphological, biochemical and 16S rRNA analysis as *Klebsiella pneumoniae* RS26. RS26 was found to be capable of N_2 fixation, ammonia production, phosphate solubilisation and IAA production. Time dependent analysis of ammonia production revealed that RS26 produced 15.21 µg/ml of NH_3 at 72 h of incubation. IAA production by the strain enhanced in presence of tryptophan and was maximum (15 µg/ml) at 48 h of incubation. Phosphate solubilisation was negatively correlated with the medium pH and maximum phosphorus solubilisation (29µg/ml) was observed after 7 days of incubation.

Keywords: PGPR, *Klebsiella pneumoniae* RS26, N_2 fixation, IAA, phosphate solubilisation

1. Introduction
The rhizospheric bacteria that can promote or enhance the plant growth through wide variety of mechanism such as N_2 fixation, IAA production, soil phosphate and zinc solubilisation, siderophore production, controlling the plant pathogens etc., are known as plant growth promoting rhizobacteria (PGPR) (Bhattacharyya and Jha, 2012) [1]. Hence, the use of chemical fertilizers, pesticides and other supplements are being replaced by the PGPR due to their great potency and environment friendly nature (Bharadwaj *et al.*, 2017) [31].

The use of chemical fertilizers in agriculture fields leads to various problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycles and destruction of biological communities. Among the micronutrient required for plant health phosphorus is an essential element. About 80% of phosphorus applied as chemicals to the agriculture field are reported to form complex with Ca^{2+}, Fe^{3+} and Al^{3+} and remains in soil as insoluble mineral form (Qureshi *et al.*, 2012) [2]. The soil insoluble phosphate can be made available to plants by utilisation of PGPR with phosphate solubilisation capacity as a sustainable and viable approach (Vessey, 2003) [3]. Several reports noted that PGPR enhance plant growth by inducing the synthesis of plant auxin (Kloepper *et al*., 2004; Yao *et al.*, 2006) [4, 5]. PGPR may also release metal-chelating siderophores into the rhizospheric soil. These siderophore then stimulate the uptake of various metal ions, including Fe, Zn, and Cu by the plants (Carrillo-Castaneda *et al.*, 2005; Egamberdiyeva 2007; Dimkpa *et al.*, 2008; Dimkpa *et al.*, 2009; Gururani *et al.*, 2012) [6-10]. In addition, PGPR also play an important role in protection of plants from pathogen by enhancing the generation of plant induced systemic resistance (ISR) (Ramamoorthy *et al.*, 2001; Kirankumar *et al.*, 2008) [11, 12]. In past, several numbers of PGPR belonging to the genera *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus and Serratia* have been isolated (Kloepper *et al.*, 1989; Okon and Labandera-Gonzalez 1994; Glick 1995; Gururani *et al.*, 2012) [9, 13-15].

We herein report the characterization of a potent PGPR, *Klebsiella pneumoniae* RS26**,** isolated from chickpea (*Cicer arietinum*) for its functional trait associated with plant growth promotion and its pathogenic nature against human.

2. Materials and methods
2.1. Isolation of PGPR strains
Soil samples from the rhizosphere of plants in the agriculture field near University of North Bengal was collected in sterilized zip bags and brought immediately to the laboratory for further processing.

1 g soils suspended in 10 ml sterilized saline water and then serially diluted up to 10^{-7}. The serially diluted samples were then spread plated into 1X nutrient agar plates and the plates were incubated at 37ºC for 24 h. The colonies were then isolated depending on the distinctive colony morphology.

2.2. Identification of the strain RS26

2.2.1. Morphological and biochemical characteristics

Morphology characterization of the bacterial isolate RS26 was evaluated by gram staining, growth pattern, motility test, and spore formation test. To separate the organism in distinguished genera gram staining was performed. Aerobic/anaerobic nature of the isolate was confirmed by growing the isolates in nutrient agar in presence of O_2, motility test was performed in the sulphide indole motility agar medium; spore formation capability was checked by malachite green staining. The biochemical characterisation studies included catalase test, Voges-Proskaur test, methyl red test, urease test, nitrate reduction test, oxidase test, citrate utilization, Indole test, starch hydrolysis test, casein hydrolysis, gelatine liquefaction test, lipid hydrolysis and fermentation of sugars (glucose, fructose, mannitol, lactose, sucrose, maltose, starch, xylose, sorbitol, mannose) (Smibert and Kreig 1994) [16].

2.2.2. Molecular identification of the RS26

2.2.2.1. Genomic DNA isolation

Genomic DNA was isolated by Murmur's method (Murmur 1961) [17]. RS26 culture was centrifuged at 8,000 rpm for 10 min at 4 ºC to collect the cell pellet. The cell pellet was washed with 0.1M EDTA: 0.15M NaCl solution (1:1) followed by centrifugation at 10,000 RPM for 5 min. The pellet was resuspended in 3 ml of 0.1 M EDTA: 0.15 M NaCl and stored at -20 ºC for 4 h. Frozen cells were then incubated at 55 ºC water bath and mixed with 50 µg/ml solution of lysozyme (prepared in 0.1 M Tris-HCl pH 8) and the mixture was incubated at 37 °C for 30 min. SDS was added to the cell lysate and incubated at 55 °C for 15 min. The resulting mixture was treated with proteinase K (4 µg/ml) for 30 min. Genomic DNA was purified from the lysate by sequential extraction with equal volume of Tris-saturated phenol (pH 8), Tris-saturated phenol: chloroform (1:1) and chloroform (Sambrook *et al.,* 1989) [18]. DNA was separated from the aqueous phase by adding double volume of 100 % ethanol followed by centrifugation at 10,000 RPM for 10 min at 4°C. The DNA pellet was air dried and dissolved in TE buffer [10 mM Tris HCl and 1 mM EDTA (pH 8.0)].

2.2.2.2. PCR amplification of 16S rRNA

Genomic DNA of RS26 was used as template for PCR amplification of 16S rRNA gene. The reaction mixture in total volume of 25 µl contained; 9.5µl ultrapure water, 5µl 5X PCR buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2µl $MgCl_2$ (2mM), 1µl dNTP's (10 mM), 1µl forward primer (10 µM) 27 F (5'AGAGTTTGATCCTGGCTCAG3'), 1µl reverse primer (10 µM) 1492R (5'TACGGTTACCTTGTTACGACTT3'), 5µl genomic DNA (20ng) and 0.50 µl DNA polymerase enzyme (5 U/µl). PCR condition was initial denatuaration step at 94 ºC for 5 min followed by 25 cycles of denaturation at 94 ºC for 30 sec, annealing at 58 ºC for 30 sec and extension at 72 ºC for 1 min and then a final extension at 72 ºC for 7 min. The PCR product was separated on 1% agarose TAE gel, cut from the gel, and then extracted and purified using gel extraction kit (QIAGEN, India). The purified PCR product was sequenced by Sanger dideoxy method.

2.2.2.3. Phylogenetic Analysis

The phylogenetic relationship of the strain RS26 was determined by comparing the 16S rRNA sequence with the sequences retrieved from the Gen Bank database of the National Center for Biotechnology Information (NCBI), via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.,* 1990) [19]. Tree was constructed by neighbour joining method using the MEGA 6 (Tamura *et al.,* 2007) [20].

2.2.3. Plant growth promoting trait of RS26

2.2.3.1. N_2 Fixation

RS26 was cultivated in Asbhy's N-free agar plates containing (g/l) mannitol, 15; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.2; $CaCl_2$, 0.2; $FeCl_3$,0.05, 10 µl Na_2MoO_4 (10% w/v) and agar-agar 20 at 37 ºC for 24 h. Ammonia production ability of the isolate was estimated by the method described by Goswami *et al.,* 2014 [21]. RS26 was inoculated and incubated in 50 ml of Asbhy's N-free liquid medium at 37 ºC for 24, 48, 72 and 96 h in separate 100 ml Erlenmeyer Flasks. After the respective time of incubation the culture broth was centrifuged at 8000 rpm for 10 min and 0.2 ml supernatant was added with 1ml Nessler's reagent and total volume was made 8.5ml by adding doubled distilled water. The mixture was incubated at 37 ºC for 30 min. Brown to yellow colour was developed indicate the ammonia production and the concentration of the ammonia was estimated by measuring the optical density at 450 nm against the standard curve prepare with 0.1-10 µmol ammonium sulphate.

2.2.3.2. Production of Indole acetic acid (IAA)

IAA production was evaluated by the method of Patten and Glick, 2002 [22]. RS26 was inoculated and incubated LB broth at 37 °C for 96 h, either in presence or absence of tryptophan (1mg/ml). After specified incubation time, the culture was centrifuged at 8000 rpm for 10 min. The culture supernatant (1ml) was mixed with 2ml of Salkowski's reagent (150 ml 98 % H_2SO_4, 7.5 ml 0.5M $FeCl_3.6H_2O$ and 250 ml distilled water) and incubated for 30 min at 25 ºC. IAA production was indicated by the development of pink colour and the concentration of the IAA was estimated my measuring the optical density of the mixture at 530 nm using the standard curve prepared with 1-20 µg/ml of standard IAA.

2.2.3.3. Inorganic Phosphate solubilisation

Qualitative estimation of phosphate solubilising ability of the isolate was done by the method of Katznelson and Bose, 1959 [23]. Bacterial culture grown in Pikovskaya broth for 24 h was spot inoculated on Pikovskaya agar plate (PAP) containing tricalcium phosphate (TCP). The plates were incubated at 37ºC for 9 days and then observed for the appearance of clear zone around the colonies at specified time period. Phosphate solubilisation index (PSI) was calculated from the following equation.

$$\text{Phosphate solubilisation index} = \frac{\text{Diameter of phosphate solubilization zone}}{\text{Growth diameter of spot inoculant}}$$

Quantitative estimation of the solubilised phosphate content was done by vanadomolybdophosphoric acid method (Barton, 1948) [24]. Bacterial culture was grown in Pikovskaya broth containing TCP for 9 days. Concentration of soluble phosphate was estimated at various incubation time using the standard curve of KH_2PO_4 (10-100 µg/ml).

2.2.4. Pathogenicity test of RS26

Pathogenecity test was done by following the method of Chahad *et al.,* 2012 [25]. The bacterial culture was grown on blood agar base supplemented with 5% (v/v) sheep blood to determine their ability to produce different types of hemolysins. Plate was incubated at 37ºC for 24 h. The results were recorded. Positive control strain was used for comparison and a clear zone on the blood agar plate was considered as a positive result.

3. Results and Discussion

The group of beneficial bacteria that enhances the plant growth and acts as biocontrol agent by wide variety of mechanisms are called plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1978) [26]. The utilization of PGPR for agriculture practices can minimize chemical inputs into soil and is also beneficial in context of increasing global concern for food and environmental quality (Verma and Shahi, 2015) [27]. Plants growth is stimulated by PGPR with various direct and indirect mechanisms. Direct mechanisms includes the acquisition of nutrient i.e. solubilisation of phosphate and zinc, nitrogen fixation, iron accumulation by siderophore and modulating the level of plant hormones etc (Patten and Glick, 1996, Glick *et al.,* 1998) [28, 29].

In the present study, *Klebsiella pneumoniae* RS26, a potent PGPR strain isolated from rhizosphere of chickpea were screened for plant growth promoting (PGP) traits and its pathogenic nature was also evaluated.

3.1. Isolation and Identification of PGPR strain RS26

Total number of bacteria isolated from the rhizospheric soil sample was 3.8 $X10^7$ CFU/ml. Among the microorganism 90 strains were further isolated depending on their distinctive colony morphology and successively purified in NA plates using streak plate methods. These isolated strains were further evaluated for their potential PGP activity. Among the bacterial isolates strain RS26 was selected for further evaluation in relation to its biochemical and molecular identification as well as its PGP activities. The strain RS26 was morphologically characterized as gram negative, rod shaped, non motile bacteria. The colony of the isolate was white, opaque, slimy, glossy, having entire margin and was round in shape. The bacterium was characterized biochemically as positive in citrate utilization, Voges-Proskauer tests, nitrate reduction, lipase and amylase production test (Table 1). Phylogenetic analysis based on 16S rRNA gene sequence comparison showed the isolate RS26 (Gen Bank accession number MH819506.1) belonging to the branch encompassing members of genus *Klebseialla* and was most closely related to *Klebsiella pneumoniae* ATCC 13884 with 99% 16S rDNA sequence similarity (Fig. 1) and hence identified as *Klebsiella pneumoniae* RS26. There are several reports on *Klebsiella pneumoniae* as potent PGPR strain, such as 4 species of *Klebsiella* having the phosphate solubilisation and auxin production activity were isolated Ji *et al.,* 2014 [30], Bhardwaj *et al.,* 2017 [31] isolated *Klebsiella pneumoniae* from the rhizosphere of *Saccharum officinarum* that was found to solubilise 17.4 µg/ml of inorganic phosphate and produced 45.32 µg/ml of IAA after 96 h of incubation. In another report the researchers isolated four PGPR strains from maize rhizosphere and identified as *Klebsiella* sp. Br1, *Klebsiella pneumoniae* Fr1, *Bacillus pumilus* S1r1 and *Acinetobacter* sp. S3r2 and all of which were found positive for N_2 fixation, phosphate solubilisation and auxin production (Kuan *et al.*, 2016) [32].

Table 1: Morphological and biochemical properties of the strain RS26

Test	Inference
Colony morphology	White, opaque, slimy, Glossy, Entire margin, round in shape
Shape	Rod
Gram Staining	-ve
Capsule	+ve
Gelatin Hydrolysis	-ve
Motility	-ve
H_2S production	-ve
Indole	-ve
MR (Methyl Red)	-ve
VP	+ve
Citrate utilization	+ve
Nitrate reduction	+ve
Fermentation of	
Arabinose	+ve
Cellobiose	+ve
Glucose	+ve
Glycerol	+ve
Lactose	+ve
Maltose	+ve
Mannitol	+ve
Mannose	+ve
Sorbitol	+ve
Sucrose	+ve
Xylose	+ve
Production of Lipase	+ve
Production of amylase	-ve
Production of Catalase	+ve
Probable Genera of RS26	*Klebseilla* sp

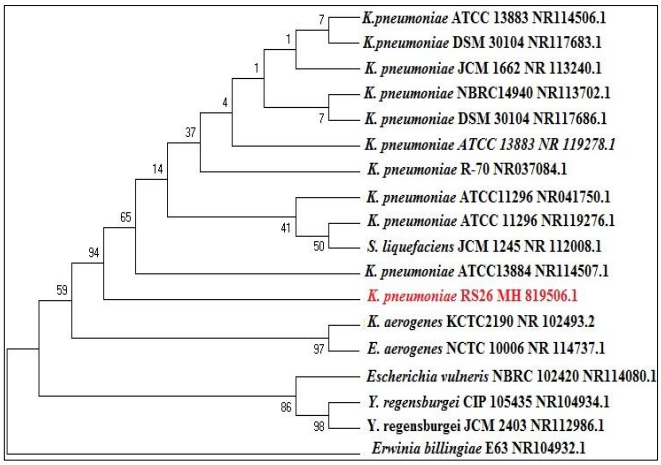


Fig 1: Phylogenetic tree construction based on 16S rRNA gene sequences reflecting the position of strain RS26 with the other *Klebsiella* species. Bar represents 1 nucleotide substitution per base. Numbers at nodes represent bootstrap values. Accession numbers are given at extreme left of each strain.

3.2. N_2 fixation and ammonia production by RS26

Symbiotic or non-symbiotic N_2 fixation by the microorganisms contribute to the N-uptake and hence to the N content of the crops (Goswami *et al.*, 2014) [21]. In our study the isolated strain RS26 was found to grow well in N-free Asbhy's media indicating their ability to fix N to ammonia. The results in Fig 2 show that RS26 produced 10.13 µg/ml of ammonia after 24 h of incubation and maximum ammonia production by the strain was recorded as 15.21 after 72 h of incubation. The strain also formed pellicle in the JNFb semi-solid N-free medium and hence was categorized as free living N fixer. Previously, various diazotrophic bacteria were isolated and characterized. They belong to the genera of *Azospirillum, Azoarcus, Enterobacter, Klebsiella and Zoogloea*. (Bilal and Malik 1987; Malik *et al.*, 1991) [33, 34]. Regulation of nitrogen fixation in *Klebsiella pneumonia* had been well documented by isolation and characterization of strains with nif-lac fusions (Douglas *et al.*, 1981) [35].

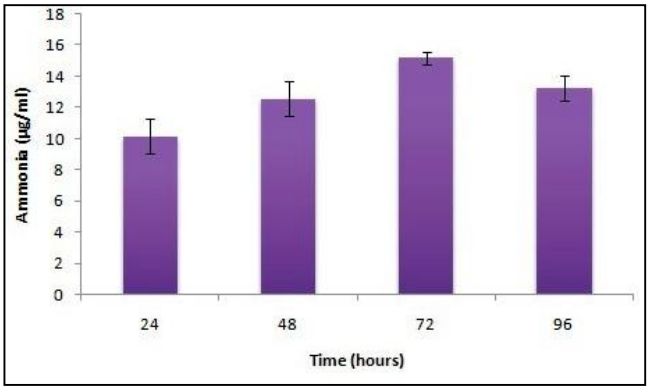


Fig 2: Ammonia production by *K. pneumoniae* RS26 in N-free Asbhy's nitrogen media. Data were represented as triplicate of mean ± SD.

3.3. IAA Production

Phytohormone IAA produced by the plants endogenously, is responsible for the root shoot elongation as well as induction of cambial cell division [22]. However, IAA produced can be effective when applied exogenously. In the present study, isolated rhizospheric bacteria were found to be producing IAA. The results in Fig 3 suggest that RS26 synthesized IAA either in presence or absence of tryptophan. Moreover, the production was more in the presence of the amino acid. Maximum IAA production of 15 and 7 µg/ml, respectively, was observed in presence and absence of tryptophan at 48 h incubation. On 96 h of incubation the production declined to 4 ug/ml and 8 ug/ml without and with tryptophan, respectively. Several bacteria have been reported to produce IAA. *Pseudomonas alcaligenes* and *Mycobacterium phlei* were found to be producing 0.3 µg/ml and 0.5 µg/ml of IAA (Egamberdiyeva *et al*. 2007) [7]. *K. pneumoniae* strains were reported for the highest production of 27.5 µg/ml (Sachdev *et al.*, 2009) [38]. Kuan *et al.*, 2016 [32] reported the production of 12.99 µg/ml of IAA by *K. pneumoniae* Fr1 which was significantly higher among the other isolated strains such as *Bacillus subtilis* UPMB (10.10 µg/ml), *Klebsiella* sp. Br1 (4.91 µg/ml), *Bacillus pumilus* S1r1 (4.55 µg/ml) and *Acinetobacter* sp. S3r2 (10.70 µg/ml). In the present research elevated production of IAA on tryptophan supplementation could be due to the amino acid being the precursor of IAA and as root exudates contain tryptophan due to the transammination and decarboxylation reactions operated in plant roots, which can stimulate PGPR strains to produce more amount of IAA (Patten and Glick, 2002) [22].

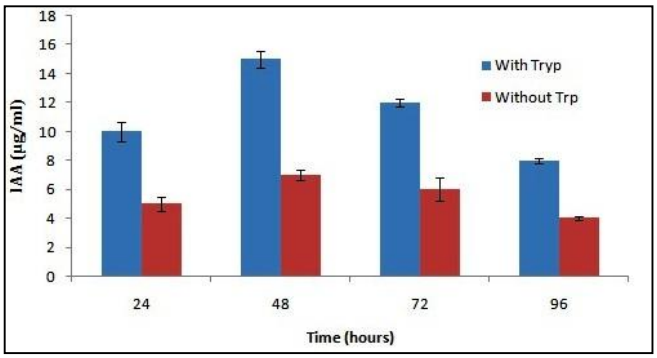


Fig 3: IAA production by *K. pneumonia* RS26 in LB broth media in presence or in absence of tryptophan. Data were represented as triplicate of mean ± SD.

3.4. Phosphate solubilisation

Qualitative and quantitative estimation of inorganic phosphate solubilisation ability of RS26 was evaluated by growing the bacterial strain in Pikovskya agar and broth media respectively, for 2, 4, 6, 7, 8 and 9 days respectively. The results in Fig 4 represent the qualitative estimation of phosphate solubilisation by the isolated strain in terms of phosphate solubilisation zone and phosphate solubilisation index (PSI). PSI was found to increase gradually from day 2 to 7 and was maximum at day 7 with a PSI of 2.09. Although phosphate solubilisation zone was maximum (11 mm) at day 8, but PSI ratio was found to be 1.77. The result also suggested that the phosphate solubilisation zone was positively correlated (r=0.91) with the diameter of the spot inoculants of the strain.

Quantitative estimation revealed that the solubilisation of the phosphate by RS26 was also increased gradually from 2 to 7 days (10-29 µg/ml) and was correlated with the decrease in medium pH from 6.4 to 5.5, further incubation decreased the amount of soluble phosphate (Fig 5). The result suggests that the strain RS26 could solubilise the phosphate by secreting some organic acids in the media as reflected by the decreased medium pH. Organic acids such as citric, propionic, gluconic, succinic and lactic acids are reported to be the most common in phosphate solubilisation (Glick *et al.*, 1998) [29]. Several scientific report suggested that the *Klebsiella* sp can solubilise the inorganic phosphates such as *Klebsiella* sp. Br1, *Klebsiella pneumoniae* Fr1 (Kaun *et al.*, 2016), *Klebsiella pneumoniae* VRE36 (Bhardwaj *et al.*, 2017) [32, 31].

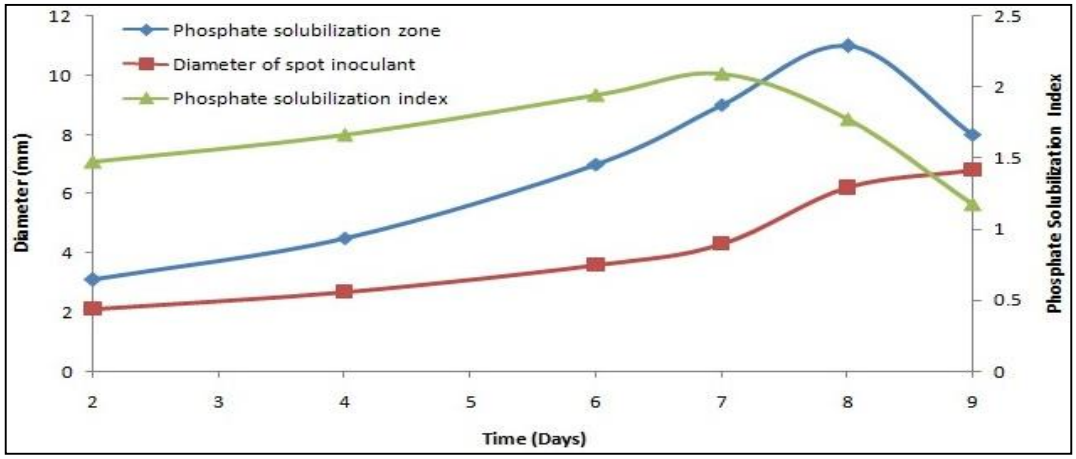


Fig 4: Evaluation of phosphate solubilization index (PSI) created by *K. pneumoniae* RS26 on Pikovskaya' sagar medium after 2, 4, 6, 7, 8 and 9 days of incubation.

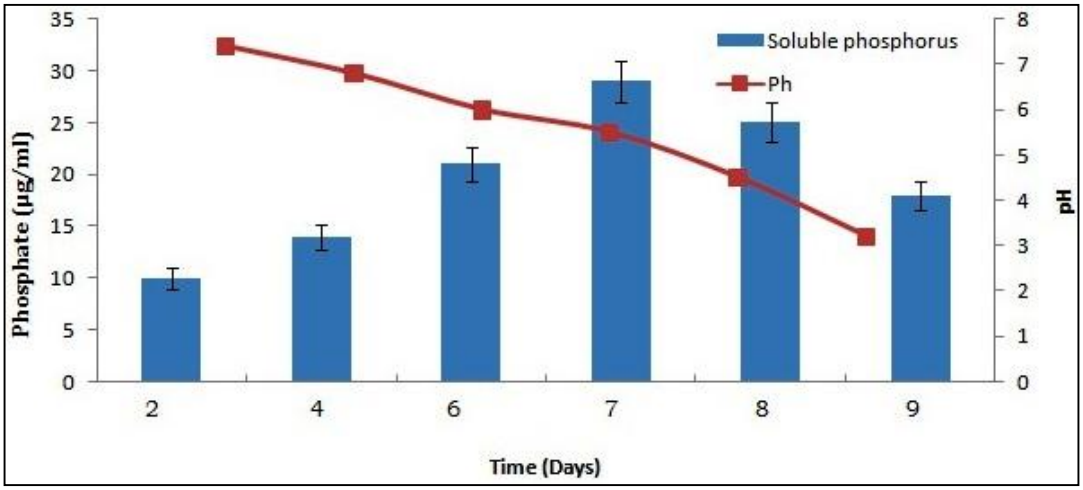
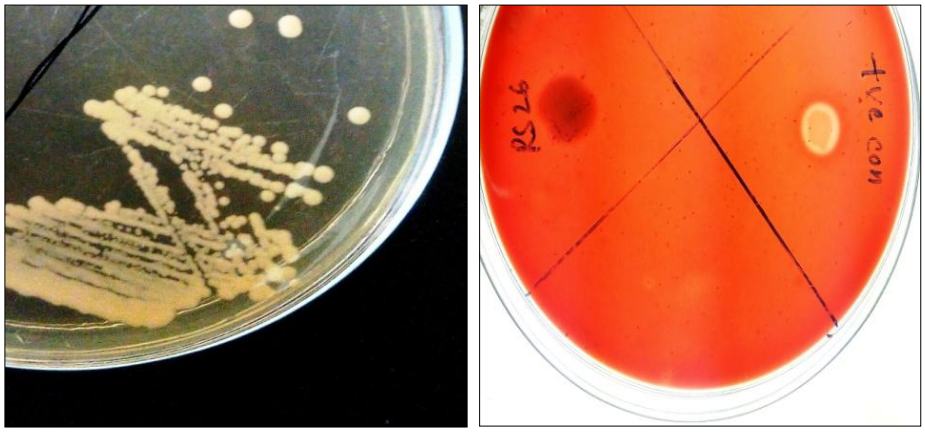


Fig 5: Quantitative estimation of soluble phosphorus by *K. pneumoniae* RS26 on Pikovskaya's broth medium after 2, 4, 6, 7, 8 and 9 days of incubation.

3.5. Pathogenecity test of RS26

Hemolysin production test of RS26 was evaluated by growing the organism in sheep blood agar medium. The result (Fig 6B) indicates the absence of the zone of hydrolysis suggesting its non-pathogenic nature. Therefore, from this experiment it can be concluded that strain RS26 can be used as PGPR for plant growth promotion without causing any human pathogenicity.



A **B**

Fig 6: Plates showing the colony morphology of RS26 on nutrient agar plate (A) and the non-haemolytic nature of RS26 as compared to the positive control (B).

4. Conclusion

In the present research a potent bacterial strain was isolated and identified as *K. pneumoniae* RS26. The strain was able to fix atmospheric N_2, produce considerable amount NH_3 and IAA, solubilize insoluble inorganic phosphate. Moreover, nonpathogenic nature of RS26 along with PGP activity makes

it as good and promising candidate for bio-fertilizer formulation. Further detailed investigations are required to check its ability of plant growth promotion in field or pot experiments.

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PLOS ONE

RESEARCH ARTICLE

Cloning and Expression of Phytase appA Gene from *Shigella* sp. CD2 in *Pichia pastoris* and Comparison of Properties with Recombinant Enzyme Expressed in *E. coli*

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Abstract

The phytase gene $appA_S$ was isolated from *Shigella* sp. CD2 genomic library. The 3.8 kb DNA fragment contained 1299 bp open reading frame encoding 432 amino acid protein ($AppA_S$) with 22 amino acid signal peptide at N-terminal and three sites of N-glycosylation. $AppA_S$ contained the active site RHGXRXP and HDTN sequence motifs, which are conserved among histidine acid phosphatases. It showed maximum identity with phytase AppA of *Escherichia coli* and *Citrobacter braakii*. The $appA_S$ was expressed in *Pichia pastoris* and *E. coli* to produce recombinant phytase $rAppA_P$ and $rAppA_E$, respectively. Purified glycosylated $rAppA_P$ and nonglycosylated $rAppA_E$ had specific activity of 967 and 2982 U mg^{-1}, respectively. Both had pH optima of 5.5 and temperature optima of 60°C. Compared with $rAppA_E$, $rAppA_P$ was 13 and 17% less active at pH 3.5 and 7.5 and 11 and 18% less active at temperature 37 and 50°C, respectively; however, it was more active at higher incubation temperatures. Thermotolerance of $rAppA_P$ was 33% greater at 60°C and 24% greater at 70°C, when compared with $rAppA_E$. Both the recombinant enzymes showed high specificity to phytate and resistance to trypsin. To our knowledge, this is the first report on cloning and expression of phytase from *Shigella* sp.

Introduction

Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis phosphate) is the major storage form of phosphorous in cereals, legumes, oil seeds and nuts [1]. Monogastric animals are incapable of digesting phytate phosphorous. Phytate also acts as an antinutritional agent, since it forms insoluble complexes with proteins and nutritionally important metal ions, such as calcium, copper and zinc and thus decreases nutrient bioavailability. The ingested phytate is largely excreted causing nutritional deficiencies and environmental pollution [1, 2].

Phytic acid is hydrolysed by phytase (myo-inositol hexakisphosphate hydrolase) to inorganic phosphate (Pi) and less phosphorylated myo-inositol derivatives [2, 3]. Phytase

Competing Interests: The authors have declared that no competing interests exist.

supplementation in animal feed increases the bioavailability of phosphorous in monogastric animals besides reducing the level of phosphorous output in their manure [4]. The enzyme is wide spread in nature, occurring in plants, animals and microorganisms. Phytases from these sources exhibit variations in structure and catalytic mechanism and consequently, have been categorized into cysteine phytases, histidine acid phosphatases (HAPs), β-propeller phytases and purple acid phosphatases [3]. Moreover, the ExPASy enzyme database (http://www.expasy.ch/enzyme/) classifies phytases into three different groups: 3-phytase (alternative name, 1-phytase; EC 3.1.3.8), 4-phytase (alternative name, 6-phytase; EC 3.1.3.26), and 5-phytase (EC 3.1.3.72). This classification is based on the carbon ring position where removal of phosphate groups from phytate is initiated [2–4].

A number of phytases have been characterized from various microorganisms such as *Aspergillus* species, *Citrobacter braakii*, *Obesumbacterium proteus*, *Bacillus subtilis*, *Escherichia coli*, *Pichia anomala*, *Erwinia carotovora* and *Yersinia intermedia* and corresponding genes have been isolated, cloned and expressed in different hosts [5–12].

Phytases belonging to HAP family have been used successfully as a feed additive. Although, the commercial production of phytase is currently focused on the fungal HAP from *Aspergillus* species, studies have suggested bacterial phytases as more promising because of their thermostability, higher substrate specificity, greater resistance to proteolysis and better catalytic efficiency. The substrate specificity property of the enzyme is highly desirable to prevent hydrolysis of other phosphate compounds so that they remain available for animal uptake [1, 2, 4].

The methylotrophic yeast *Pichia pastoris* has been successfully used as a host for heterologous gene expression, producing high level of recombinant proteins, including phytase. *P. pastoris* can grow in simple defined media, reach a very high cell density, and accumulates extremely high concentration of intra- or extracellular protein under the control of the *AOX1* promoter. In addition, *P. pastoris*, as a eukaryotic expression system, can carry out protein processing, folding, and posttranslational modifications [13, 14].

In our previous communication, we reported purification and characterization of phytase from *Shigella* sp. CD2 [15]. We herein report molecular cloning and sequencing of the phytase gene from *Shigella* sp. CD2 and its extracellular expression in *P. pastoris* strain GS115. The characteristic properties of the enzyme were compared with that expressed in *E. coli* strain BL21 (DE3).

Materials and Methods

Strains, plasmids and chemicals

The bacterial strain used in this study *Shigella* sp. CD2 (Accession no. FR745402) was isolated from wheat rhizosphere. The pUC18 vector, pGEM-T vector system, *E. coli* XL1 Blue and PCR reagents were purchased from Promega, USA. Restriction enzymes, Endo H deglycosylase and T4 DNA ligase were from New England Biolabs (Beverly, MA). *E. coli* BL21(DE3) and pET-20b(+) vector (Novagen, Madison, WI) and MagicMedia^{TM} *E.coli* Expression Medium (Invitrogen, San Diego,CA) were used for bacterial expression. The expression medium has two components, (a) Ready to use medium and (b) IPTG solution. For expression in eukaryotic system, *P. pastoris* GS115(*his4*) and pPIC9 expression vector were purchased from Invitrogen, San Diego, CA. Plasmid pPIC9 contains the promoter and terminator of the *P. pastoris AOX1* gene, the α-mating factor prepro-secretion signal from *S. cerevisiae* and the HIS4 auxotrophic selection marker for transforming *P. pastoris* GS115. Regeneration dextrose base (RDB), buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY) media were prepared according to the manual of the *Pichia* Expression kit (Invitrogen, San Diego, CA). All

other chemicals and microbiological media were from Sigma Chemical Company, USA; E. Merck, Germany; and HiMedia Laboratory, India.

Cloning of the phytase gene and nucleotide sequence analysis

Genomic DNA isolated from *Shigella* sp. CD2 [16] was partially digested with *Eco*RI to obtain 3 to 6 Kb fragments. The fragments were cloned in *Eco*RI site of pUC18 vector and transformed into *E.coli* XL1 Blue. The transformants were screened for phytase activity on LB-agar plates containing 100 μg mL^{-1} ampicillin and 1% sodium phytate. Phytase positive clones formed phytate clearance zone around the colony. The recombinant plasmid (pUCphy) was isolated from phytase positive clone with highest clearance zone; the 3.8kb insert in the plasmid was sequenced by using vector specific M13-pUC forward (5'- GTTTTCCCAGTCACGAC-3') and reverse (5'-CAGGAAACAGCTATG-3') primers and putative phytase encoding ORF was identified. The amino acid sequence encoded by the ORF was analyzed for the presence of signal peptide by SignalP 4.1 Server(http://www.cbs.dtu.dk/services/SignalP) [17] and for disulphide bond in the tertiary structure by using Softberry CYS_REC online services (www.softberry.com). Mature phytase gene without the signal sequence was amplified from pUCphy by using internal primers, PhyF (5'-ATGAATTCGCTCAGAGTGAGCCGGAG-3' with 5' *Eco*RI restriction site) and PhyR (5'GATGCGGCCGCCAAACTGCACGCCGGTATG-3' with 5' *Not*I site). The PCR product was cloned in pGEM-T vector following manufacturer's instruction and sequenced using T7 and SP6 universal primers. Homology search in GenBank was done using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) [18]. The amino acid sequence of the cloned gene was deduced and then aligned by ClustalW program (http://www.ebi.ac.uk/clustalW) [19]. The phylogenetic analysis of the protein was performed by neighbour joining method using MEGA 4 [20]. Bootstrap analysis was used to evaluate the tree topology of the neighbour joining data by performing 500 replicates. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The recombinant pGEM-T vector harboring the phytase gene was named pGEMT-$appA_S$.

Construction of *P. pastoris* and *E. coli* expression plasmids and transformation

Two different plasmids were constructed for expression of $appA_S$ in *P. pastoris* GS115 and *E. coli* BL21(DE3). For *P. pastoris* expression, the pGEMT-$appA_S$ plasmid was cut with *Eco*RI and *Not*I. The resulting 1.2 kb DNA fragment was ligated into pPIC9 digested with *Eco*RI and *Not*I to generate pPIC9-$appA_S$. The pPIC9-$appA_S$ linearized with *Bsp*E1 was transformed into *P. pastoris* GS115 by the spheroplasting protocol according to the manual of the *Pichia* Expression kit (Invitrogen, San Diego, CA) and transformants were selected for ability to grow on histidine-deficient medium. The his^+ transformants were further screened for Mut^+ and Mut^S phenotypes. The integration of the expression cassette into the genome of *P. pastoris* GS115 was ascertained by PCR using the 5' *AOX1* and 3' *AOX1* primers. For expression in *E. coli*, the 1.2 kb fragment released from the pGEMT-$appA_S$ plasmid was ligated into pET-20b(+) to generate the construct pET-20b(+)-$appA_S$, which was transformed into *E. coli* BL21(DE3) and transformants were selected in presence of 100 μg mL^{-1} ampicillin.

Expression of $appA_S$ in *P. pastoris* GS115

The Mut^+, pPIC9-$appA_S$ transformed *P. pastoris* GS115 was inoculated into 10 mL of YPD (1% yeast extract, 2% peptone and 2% dextrose) and incubated overnight at 30°C and 300 rpm shaking. 1mL of starter culture was transferred to 100 mL of BMGY medium and grown at

30°C and 300 rpm shaking until culture reached an OD_{600} of 1. Cells were subsequently harvested by centrifugation at 2100×g for 5 min and used to inoculate 100 mL of BMMY medium containing 0.5% methanol as inducer. The culture was incubated at 30°C and 300 rpm shaking for 96 h and the induction was maintained by adding 0.5% (v/v) methanol at every 24 h intervals. Extracellular and periplasmic phytase activity and medium pH were monitored at every 12 h intervals. For isolation of extracellular fraction, the culture was centrifuged at 2100×g for 5 min and the cell free medium was concentrated and diafiltered by Vivaspin-20 (30 kDa cut-off) sample concentrator (GE Healthcare, UK). For periplasmic fraction isolation, cell pellet was submitted to 5 cycles of freezing (-20°C for 2 h) and thawing (28°C for 1 h), followed by extraction with 100 mM acetate buffer (pH 5.5) at 28°C in a rotatory shaker (100 rpm). The extracted sample served as periplasmic fraction. Induction of $appA_S$ expression was determined by 12% SDS-PAGE analysis of the extracellular fraction. *P. pastoris* GS115 transformed with pPIC9 vector served as control. Recombinant protein produced by $appA_S$ in *P. pastoris* GS115was named $rAppA_P$.

Expression of $appA_S$ in *E. coli* BL21(DE3)

Expression of $appA_S$ in *E.coli* BL21(DE3) was analysed by using MagicMedia™ *E.coli* Expression Medium following manufacturer's instruction. *E. coli* BL21 (DE3) cells transformed with pET-20b(+)-$appA_S$ was grown overnight in LB medium at 37°C and 200 rpm shaking. The culture at 1% (v/v) was inoculated into the MagicMedia (19:1, ready to use medium: IPTG solution) and grown overnight at 37°C and 300 rpm shaking. The cells were then harvested by centrifugation at 11,200×g for 10 min, suspended in 50 mM acetate buffer (pH 5.5), disrupted by sonication and centrifuged. The supernatant and the pellet dissolved in 50 mM acetate buffer (pH 5.5) served as soluble and pellet fractions, respectively. Induction of $appA_S$ expression in both the fractions was determined by 12% SDS-PAGE. Both the fractions were also checked for phytase activity. *E. coli* BL21 (DE3) transformed with pET-20b(+) vector was used as control. Recombinant protein produced by $appA_S$ in *E.coli* BL21 (DE3) was named $rAppA_E$.

Protein estimation and SDS-PAGE analysis

Total protein concentration was determined by the dye binding assay of Bradford using bovine serum albumin (BSA) as standard [21]. SDS-PAGE analysis was performed with 12% polyacrylamide gel according to the method of Laemmli [22]. After electrophoresis, the gel was stained with CBB R-250 reagent (0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid and 40% methanol) and then destained. Broad range pre-stained protein standards were used as markers.

Purification of $rAppA_E$ and $rAppA_P$

Recombinant $rAppA_P$ was purified from the cell free medium of pPIC9-$appA_S$ transformed *P. pastoris* GS115 culture induced with methanol for 60 h. The concentrated and diafiltered cell-free medium was loaded on to CM-cellulose column and bound proteins were eluted by 50 mM acetate buffer (pH 5.5) with linear gradient of 0–0.5 M NaCl. The active fractions were pooled for subsequent studies. For purification of $rAppA_E$, the IPTG induced culture of pET-20b(+)-$appA_S$ transformed *E.coli* BL21 (DE3) was harvested by centrifugation at 11,200×g for 10 min. The cell pellet was suspended in 50 mM acetate buffer (pH 5.5), disrupted by sonication and centrifuged. The supernatant was loaded onto a Ni- Sepharose Fast Flow column (2 x 5 cm, GE Healthcare, UK) pre-equilibrated with 50 mM acetate buffer (pH 5.5) containing 10 mM imidazole. The bound proteins were eluted with 50 mM acetate buffer (pH 5.5) containing 100 mM imidazole. Fractions with phytase activity were pooled for subsequent studies.

Determination of phytase activity

Phytase activity was determined as described previously [15]. The reaction mixture in a final volume of 2 mL contained, acetate buffer (pH 5.5), 100 mM; sodium phytate, 2 mM; and 100 μL enzyme preparation. The reaction was carried out at 37°C for 30 min followed by termination of reaction by adding 2 mL of 10% trichloroacetic acid. The released Pi was measured spectrophotometrically by adding 2 mL of ammonium molybdate (0.5%), sulphuric acid (5 N) and ascorbic acid (2%) solution. One unit (U) of phytase activity represents 1 μmol of Pi released min^{-1} under assay conditions.

Characterization of $rAppA_E$ and $rAppA_P$

The pH optima was determined by measuring enzymatic activity at pH 2.5–8.5 in the following buffers (50 mM): glycine-HCl (pH 2.5 and 3.5), sodium acetate (pH 4.5 and 5.5), and Tris-HCl (pH 6.5, 7.5 and 8.5). The optimum temperature for activity was determined at temperatures ranging from 10 to 80°C. Thermostability of the enzyme was determined by preincubating the purified enzyme at 10 to 80°C for 30 min followed by measuring phytase activity under standard conditions. To study the effect of metal ions and salts (2 mM), phytase activity was monitored in presence of $CaCl_2$, $MnSO_4$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $CuSO_4$ and EDTA. To determine the susceptibility to digestive proteases, the 50 U of purified $rAppA_E$ or $rAppA_P$ was preincubated with pepsin and trypsin (30 U, Sigma) at 37°C and phytase activity was monitored 30 min later.

Substrate specificity of the enzyme was determined by replacing sodium phytate in the standard reaction mixture of various pH (pH 4.5–7.5) with an equal concentration (2 mM) of either of phosphorylated compounds, such as p-nitrophenyl phosphate (pNPP), ATP, ADP, disodium pyrophosphate (dSPP), D-glucose-6-phosphate (G6P) and D-fructose-6-phosphate (F6P). K_m for phytate was determined using the Lineweaver-Burk plot. K_{cat} values for both the enzymes were also determined.

Deglycosylation

The deglycosylation of $rAppA_P$ was carried out using Endo H deglycosylase (New England Biolabs) following manufacturer's instruction. The reaction mix containing, 50 U of purified $rAppA_P$, 600 μL of 50 mM Tris buffer (pH 7.0) and 10 U of Endo H in final volume of 1 mL, was incubated at 37°C for 2 h. N-glycosylation was determined by assessing the migration shift of Endo H treated $rAppA_P$ in 12% SDS-PAGE.

Western blot analysis

For immunoblot analysis, purified $rAppA_E$ and deglycosylated $rAppA_P$ proteins separated by 12% SDS-PAGE, were transferred to polyvinylidenedifluoride (PVDF) membrane by semi-dry method using Electroblotting apparatus (Atto, Japan). Purified rabbit antibody raised against *E. coli* phytase, diluted 1:1000 prior to application, was the primary antibody. The reacted polypeptide was visualised with a secondary antibody, goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range prestained standards were used as markers.

Results

Isolation of gene encoding phytase from the genomic library

For cloning of phytase gene, a size selected genomic library of *Shigella* sp. CD2 was constructed in pUC18 vector using *Eco*RI digested genomic DNA. The library was screened for phytase

activity based on formation of clearance zone in phytate-agar medium. Among the six phytase positive clones, one with highest phytate clearance zone and phytase activity in the cell lysate was selected. The clone harbouring plasmid pUC18-phy had DNA insert of 3.8 kb. Sequence analysis of the insert indicated presence of an open reading frame (ORF) of 1299 bp, encoding a protein of 432 amino acids (Fig 1).

Sequence and phylogenetic analysis

Homology analysis of deduced amino acid sequence by BLAST program revealed 98 and 62% similarity with AppA phytase of *E. coli* and *C. braakii*, respectively. Hence, *Shigella* sp. CD2 phytase ORF was named as $appA_S$ and the encoded protein as $AppA_S$. The nucleotide sequence was deposited in the GenBank under accession number FR865899. $AppA_S$ contained three potential sites of N-glycosylation, a putative signal peptide of 22 amino acids at N-terminal end and 8 cysteine residues among which 99–130, 200–210, 404–413 were the most possible disulphide bond pairs. The calculated molecular mass of the protein with and without the signal sequence were about 47 and 45 kDa, respectively. Alignment of $AppA_S$ with other enteric bacterial phytases in the GenBank using ClustalW program showed presence of N-terminal RHGXRXP motif, C-terminal HDTN motif and five conserved cysteine residues. $AppA_S$ and *E. coli* AppA differed in sequence at six positions; $AppA_S$ contained P, Q, N, K, K, T in place of S, R, K, E, M, A in *E. coli* AppA at positions 102, 190, 202, 208, 298, 299, respectively (Fig 2). A phylogenetic tree was constructed based on the alignment using the neighbour joining method.

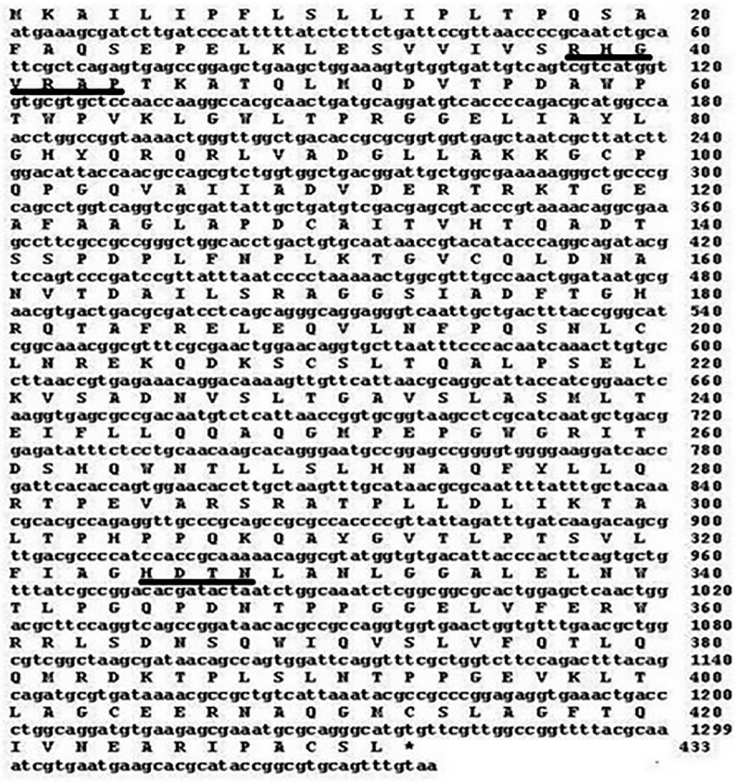

Fig 1. Nucleotide (1–1299) and deduced amino acid sequences (432) of the putative phytase gene $appA_S$, from *Shigella* sp. CD2. The conserved HAP family active site motifs are underlined. Stop codon is shown by asterisk.

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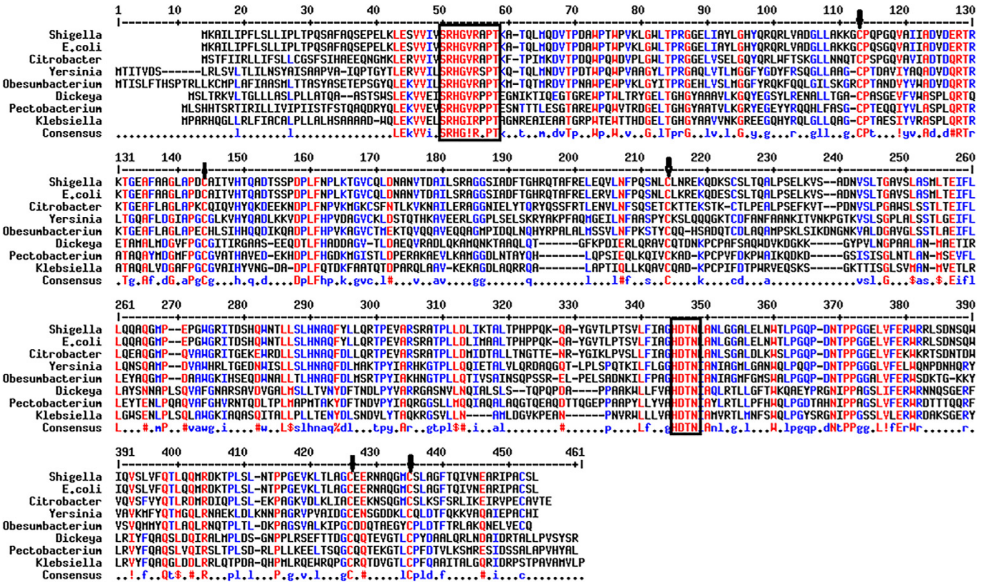

Fig 2. Multiple alignment of homologs of the *Shigella* sp. CD2 phytase AppA$_S$. Conserved active site motifs are boxed and conserved cysteine residues are shown by arrows. The source and GenBank Accession Nos. of proteins are: *Shigella* sp. CD2, CCA94903; *Escherichia coli* AppA, EDX38944; *Dickeya paradisiaca*, ABW76125; *Klebsiella pneumoniae* ASR1, AAM23271; *Yersinia intermedia*, ABI95370; *Citrobacter braakii*, AAS45884; *Obesumbacterium proteus*, AAQ90419; *Pectobacterium carotovorum* subsp. *carotovorum*, ABY76184.

doi:10.1371/journal.pone.0145745.g002

The topology of the phylogram also confirmed AppA$_S$ to be closely related to AppA phytase of *E. coli* and *C. braakii* (Fig 3).

Expression of appA$_S$ in *P. pastoris* G115

The appA$_S$ was cloned in *Eco*RI and *Not*I sites of *P. pastoris* expression vector pPIC9. The recombinant plasmid pPIC9-appA$_S$ carried the appA$_S$-expression cassette consisting of 1.2 kb appA$_S$ gene in frame with *S. cerevisiae* α-factor secretion signal, flanked by *AOX1* promoter and terminator sequences. Transformation of linearized pPIC9-appA$_S$ into *P. pastoris* GS115 gave about 20 his$^+$ transformants. The integration of appA$_S$-expression cassette into the host genome was ascertained by PCR using 5' and 3' *AOX1* primers. PCR amplification products of about 0.5kb and 1.7 kb in pPIC9 transformed and pPIC9-appA$_S$ transformed *P. pastoris* GS115, respectively, indicated the integration of appA$_S$-expression cassette into the genome of the later.

The pPIC9-appA$_S$ transformed *P. pastoris* GS115 colonies were screened for Mut phenotypes, and for extracellular and periplasmic phytase activity. A Mut$^+$ colony with highest extracellular phytase activity was selected for shake flask expression. At 60 h of methanol induction, the selected transformant showed maximum extracellular recombinant phytase (rAppA$_P$) production of 62 U mL^{-1} with specific activity 477 U mg^{-1} and an extracellular protein concentration of 0.13 mg mL^{-1}. SDS-PAGE analysis of concentrated and diafiltered cell-free extract showed two protein bands of approximate molecular mass 59 and 65 kDa (Fig 4A). Deglycosylation of rAppA$_P$ by Endo H deglycosylase resulted in single band of apparent molecular mass 45kDa (Fig 4B).

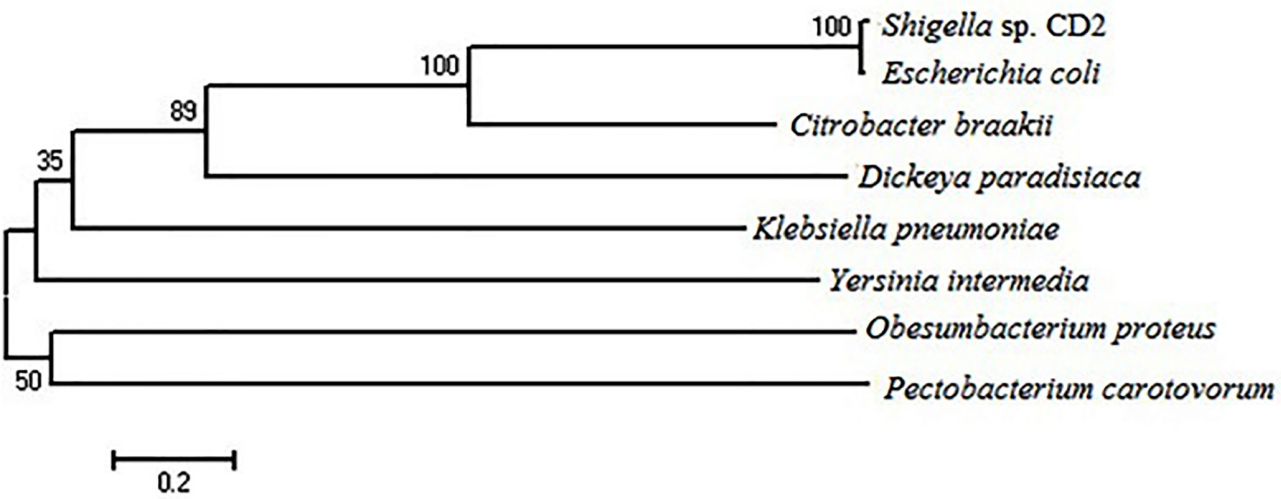

Fig 3. Phylogenetic tree of homologs of the *Shigella* sp. CD2 phytase AppA$_S$. The bar represents 2 substitutions per 10 amino acids. GenBank Accession Nos. are as in Fig 2 legend.

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Expression of appA$_S$ in *E. coli* BL21(DE3)

The mature appA$_S$ was cloned into *E. coli* expression vector pET-20b(+) and the recombinant plasmid pET-20b(+)-appA$_S$ was transformed into *E. coli* BL21(DE3). The transformant was induced in MagicMedia supplemented with IPTG and after overnight induction cells were disrupted by sonication. Recombinant phytase (rAppA$_E$) overexpression in the soluble and pellet fractions of sonicated cells was analyzed by SDS-PAGE. As shown in the results of Fig 5A, the soluble fraction of the induced cell exhibited protein overexpression band of approximately 45kDa, which agrees with the predicted molecular weight deduced from the amino acid sequence of AppA$_S$. Phytase activity in the soluble fraction was 176 U mL^{-1} (specific activity 568 U mg^{-1}), whereas negligible activity was detected in the pellet fraction. The results thus indicate a correlation of rAppA$_E$ overexpression with phytase activity. Western blot analysis of rAppA$_E$ and deglycosylated rAppA$_P$ using rabbit polyclonal antibody against *E. coli* AppA further demonstrated that the specific band with apparent molecular mass of 45 kDa was recombinant phytase (Fig 5B).

Purification and properties of rAppA$_E$ and rAppA$_P$

Recombinant rAppA$_P$ was purified by cation exchange chromatography of diafiltered extracellular fraction of methanol induced *P. pastoris* GS115 culture transformed with pPIC9-appA$_S$ and rAppA$_E$ was purified from the soluble fraction of pET-20b(+)-appA$_S$ transformed *E. coli* BL21 (DE3) using Ni-Sepharose Fast Flow affinity chromatography. Purified rAppA$_P$ and rAppA$_E$ had specific activities of 967 and 2982 U mg^{-1}, with recovery of 75 and 83%, respectively. The results of biochemical properties of rAppA$_P$ and rAppA$_E$ are shown in Table 1. Compared with the glycosylated rAppA$_P$, the nonglycosylated rAppA$_E$ was more active at pH 3.5–7.5. Both the enzymes had more than 50% activity in the pH range 3.5 to 6.5 with pH optima at 5.5 (Fig 6A). Both rAppA$_E$ and rAppA$_P$ had temperature optima of 60°C. Compared with rAppA$_P$, rAppA$_E$ had 11 and 18% greater relative activity at 37 and 50°C, respectively, whereas at higher incubation temperature rAppA$_P$ was more active than rAppA$_E$ (Fig 6B). For determination of thermal stability, the purified rAppA$_E$ or rAppA$_P$ were pre-incubated at 10 to 80°C for 30 min

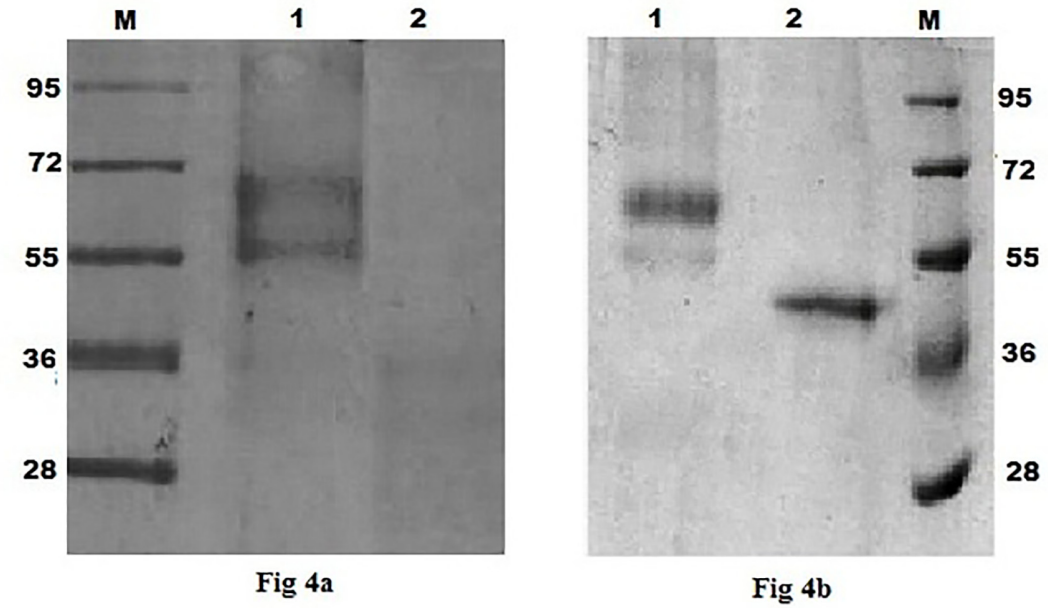

Fig 4. (a) SDS-PAGE analysis of rAppA$_P$ expressed in *P. pastoris* GS115. Lane, M-molecular weight markers, 1-extracellular fraction of *P. pastoris* GS115 transformed with pPIC9-appA$_S$, 2- extracellular fraction of *P. pastoris* GS115 transformed with pPIC9. (b) SDS-PAGE analysis of glycosylated and deglycosylated rAppA$_P$. Lane, 1- glycosylated rAppA$_P$, 2- deglycosylated rAppA$_P$, M- molecular weight markers.

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and then assayed for enzymatic activity. Although, the two enzymes didn't differ in their thermostability in the temperature range 10 to 50°C, rAppA$_P$ was more thermotolerant at higher

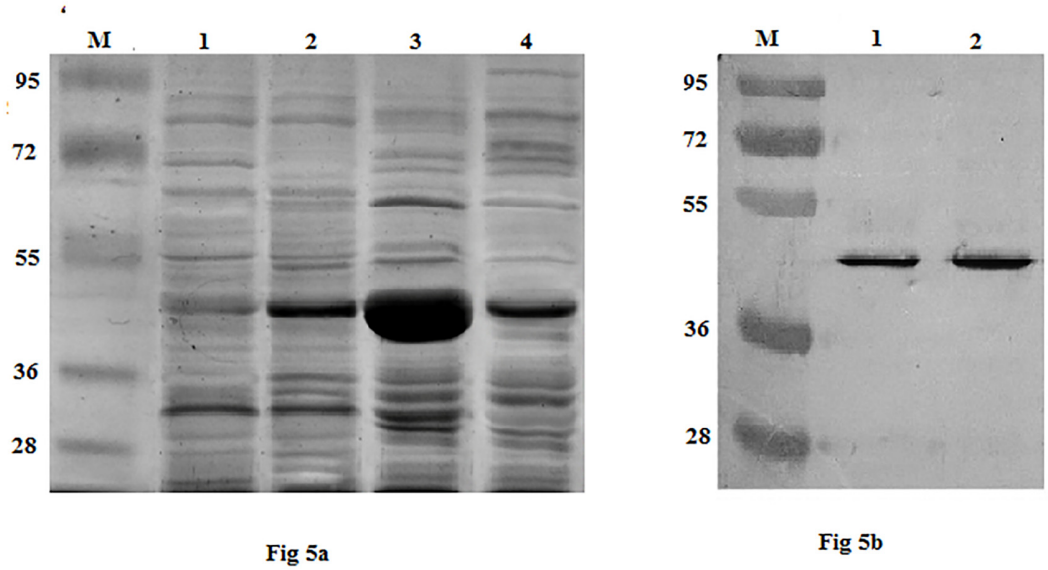

Fig 5. (a) SDS-PAGE analysis of rAppA$_E$ expressed in *E. coli* BL21(DE3). Lane, M-molecular weight markers, 1- soluble fraction of induced BL21 transformed with pET20b(+), 2-pellet fraction of induced BL21 transformed with pET20b(+), 3- soluble fraction of induced BL21 transformed with pET-appA$_S$, 4- pellet fraction of induced BL21 transformed with pET-appA$_S$. (b) Western blot analysis. Lane, M- Molecular weight marker, 1- purified rAppA$_E$, 2-purified and deglycosylated rAppA$_P$.

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Table 1. Properties of $rAppA_E$ and $rAppA_P$.

Properties	Results	
	$rAppA_E$	$rAppA_P$
*Substrate specificity (Sodium phytate)	100%	100%
K_m for phytate (mM)	0.18	0.22
V_{max} (μmol min^{-1})	149.1	48.35
K_{cat} (Sec^{-1})	2.23×10^3	0.72×10^3
K_{cat}/K_m (Sec^{-1} mM^{-1})	12.43×10^3	3.23×10^3
Specific activity of purified enzyme (U mg^{-1} protein, 37°C)	2982	967
Temperature optima (°C)	60	60
pH optima	5.5	5.5
$Thermostability (%)	100	100
#Activity in presence of trypsin	70%	65%
#Activity in presence of pepsin	55%	50%
Activity in presence of metal ions (20 mM): Ca^{2+}	130%	105%
Mg^{2+}	125%	110%
Mn^{2+}	109%	102%

*Activity in presence of ATP, ADP, pNPP, dSPP, G6P, F6P was negligible.

$Activity after pre-incubation of enzyme at 40°C for 30 min.

Recombinant enzyme (50 U) was pre-incubated with pepsin or trypsin for 60 min followed by determination of phytase activity.

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temperature. Consequently, at 60 and 70°C $rAppA_P$ had 33 and 24% higher activity in comparison to $rAppA_E$, respectively (Fig 6C). K_m values for phytate as determined by Lineweaver-Burk plot were 0.18 and 0.22 mM for $rAppA_E$ and $rAppA_P$, respectively (Table 1). The K_{cat} value for $rAppA_E$ was 2.23×10^3 $sec^{-1}$and for $rAppA_P$ was 0.72×10^3 sec^{-1}.

Both $rAppA_E$ and $rAppA_P$ were highly specific to the substrate, sodium phytate. Activity with either of phosphorylated substrates, such as ATP, ADP, pNPP, dSPP, G6P or F6P was negligible. The relative phytase activities of $rAppA_E$ and $rAppA_P$ were enhanced up to 130% in presence of Ca^{2+}, Mg^{2+} and Mn^{2+}, whereas Cu^{2+}, Fe^{2+}, Zn^{2+} or EDTA showed inhibitory effect. To determine the protease resistance the purified recombinant phytases (50 U) were pre-incubated separately with 30U of either pepsin or trypsin at 37°C. The $rAppA_E$ and $rAppA_P$ retained 70 and 65% activity on treatment with trypsin, and 55 and 50% of activity on treatment with pepsin, respectively, indicating greater resistance to trypsin.

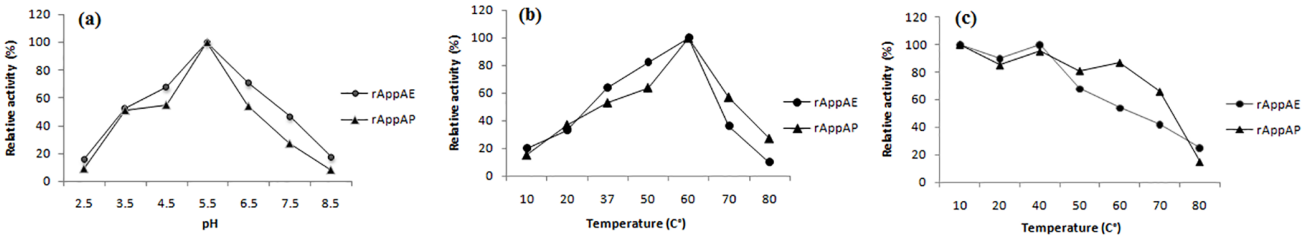

Fig 6. Characterization of purified $rAppA_E$ and $rAppA_P$. (a) pH profile (b) Temperature profile and (c) Thermal stability. Results of phytase activity represent the mean of three independent values.

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Discussion

The phytase structural gene ($appA_S$) from *Shigella* sp. CD2 had an ORF of 1299 bp encoding 432 amino acid protein ($AppA_S$) containing N-terminal 22 amino acid signal peptide, three probable disulphide bridges and three sites of N-glycosylation. Presence of the signal peptide and disulphide bridges indicates the periplasmic localization of the native protein. $AppA_S$ showed significantly high homology with AppA phytase of *E. coli* and *C. braakii* suggesting that the proteins may have similar structure and mechanism of action. Moreover, all these AppA phytases form a separate branch in phylogenetic tree. As in the present study, phytase AppA from *C. braakii* was more closely related to the *E. coli* AppA than to other phytases [6]. $AppA_S$ contained the conserved N-terminal RHGXRXP and C-terminal HD active site motifs, and six conserved cysteine residues, which are characteristics of phytase belonging to the HAP family [23]. Till date, seven genera of family enterobacteriaceae have been reported to produce phytase, and relevant genes have been cloned and all of them belong to HAP family [6, 7, 11, 12, 23].

The expression of enzyme as secreted protein is one of the useful and important characteristics for its economical production in industry. *P. pastoris* has been successfully used as host organism for extracellular production of recombinant proteins at high level, including phytase [2, 5, 6, 10, 11]. Phytase $appA_S$ was expressed in *P. pastoris* to produce $rAppA_P$ as extracellular protein with highest activity (62 U mL^{-1}) at 60 h of methanol induction, with specific activity of 477 U mg^{-1}. The $rAppA_P$ activity is higher than that of *phy*C gene encoding neutral phytase expressed in *P. pastoris* (12.5 U mL^{-1}) [2]. However, the yield is lower than that of AppA phytase of *C. braakii* (197 U mL^{-1}) and *E. coli* (112.50 U mL^{-1}) [6, 9]. The lower activity of $rAppA_P$ might be due to observed increase in the medium pH above 7 during cultivation of *P. pastoris*. This could be confirmed by significant decrease in activity of purified $AppA_P$ at pH >7 (Fig 6A). The expression level and activity of $rAppA_P$ could be increased further by optimization of bioprocess and control of medium pH at <7. Moreover, the reduced phytase activity could also be due to the variation in codon usage between *Shigella* sp. and *P. pastoris*. Previous studies have shown the effect of codon bias on expression and activity of recombinant phytase and other enzymes [14, 24]. Xiong *et al*. used *P. pastoris* preferred codons and modified signal sequences to improve the expression of heterologous phytase from *Peniophora lycii* by 13.6 fold [14]. Similarly, extracellular expression of *phy*C gene from *B. subtilis* WHNB02 in *P. pastoris* yielded 2.40 U mL^{-1} phytase. Synthesis of *phy*C according to *P. pastoris* codon usage without altering the protein sequence enhanced activity by about 8 folds to 18.50 U mL^{-1} [24]. The recombinant $rAppA_P$ was expressed as multiple proteins of higher molecular weights, which on deglycosylation produced protein of about 45 kDa, similar to that of $rAppA_E$, indicating post translational glycosylation of the recombinant protein in *Pichia* system (Fig 4A and 4B). As in the present study, SDS-PAGE analysis of recombinant AppA from *E. coli* expressed in *P. pastoris* appeared as diffused band of molecular size 55 kDa, however, a sharp band was observed after the purified phytase was deglycosylated [9]. Similarly, AppA from *C. braakii* expressed in *Saccharomyces cerevisiae* migrated as a broad diffusion band (110–160 kDa) in SDS-PAGE gel due to extensive N-linked glycosylation, while the same protein expressed in *E. coli* had molecular size of 49 kDa [6].

To examine the effect of glycosylation on enzymatic properties of $rAppA_P$, $appA_S$ was also expressed in *E. coli* to produce $rAppA_E$. The periplasmic signal sequence was removed for targeting the enzyme to the intracellular space in order to avoid the possibility of contamination of recombinant enzyme preparation with two native periplasmic AppA phytases in the host cell [25]. Phytase activity of nonglycosylated $rAppA_E$ was 176 U mL^{-1} (specific activity 568 U mg^{-1}). The $rAppA_E$ activity is significantly higher than that of *phy*A gene of *O. proteus* (9.6 U

mg^{-1}) and *app*A gene of *E. coli* (17.1 U mg^{-1}) expressed in *E. coli* as intracellular proteins [7]. Most of the other studies on expression of recombinant phytase in *E. coli* have shown accumulation of phytase as inclusion body in the cell [6, 26].

The purified $rAppA_P$ and $rAppA_E$ had specific activities of 967 and 2982 U mg^{-1}, respectively. The difference in glycosylation between the two enzymes partially affected their biochemical properties. Both the recombinant enzymes had pH optima of 5.5 and more than 50% of activity was maintained between pH 3.5 to 6.5. The pH optimum of most of the enterobacterial phytase AppA is in the range of 4.5 to 5.5. The enzyme from *E. coli*, *O. proteus*, *C. braakii*, *Y. intermedia*, and *E. carotovora* showed optimum pH of 4.5, 4.9, 5.0, 4.5 and 5.5, respectively [6,7, 9,11,12]. Although $rAppA_P$ and $rAppA_E$ shared the same optimal temperature of 60°C, the former was more active at 70 and 80°C. As in the present study, the temperature optima of other reported bacterial AppA phytases were in the range of 40–65°C [7, 9,11,12]. Glycosylated $rAppA_P$ had improved thermotolerance, especially at higher temperatures of 60 and 70°C over that of $rAppA_E$. The K_m values of 0.18 mM for $rAppA_E$ and 0.22 mM for $rAppA_P$ are less than that of the phytases from *O. proteus*(0.34 mM), *E. coli* (0.55 mM), *E. carotovora* (0.25 mM), *K. pneumoniae* (0.28 mM) [7, 9,12, 27], but higher than that of the phytase from *Y. intermedia* (0.125 mM) [11]. The catalytic efficiency of $rAppA_E$ was found to be much higher than that of $rAppA_P$ as reflected by their K_{cat} values.

Glycosylaion is one of the most important post translational modifications that affects protein function and properties. Previous studies have shown the influence of N-glycosylation on biochemical properties of proteins, such as molecular mass, isoelectric point, surface charge distribution and thermotolerance [3, 28]. As in the present study, increased level of glycosylation of phytase from *A. fumigatus* expressed in *P. pastoris* improved the thermotolerance of the protein over the deglycosylated form [28]. Similarly, phytase from *C. braakii* expressed in *S. cerevisiae* retained 50% higher activity upon heat treatment at 70°C for 30 min as compared to *E. coli* expressed protein [6]. Although there are very few studies on effect of glycosylation on K_m, recently Yao *et al.* reported an alteration in K_m of recombinant *E. coli* AppA phytase on enhancement of glycosylation. The K_m values for WT, Q258N mutant and Q258N/Q349N mutant were 0.48, 0.53 and 0.43 mM, respectively [29]. Phytase in the present study was highly specific to the substrate phytate as observed for AppA phytase from *E. carotovora* and *Y. intermedia* [11,12], whereas phytase from *E. coli* and *O. proteus* also cleaved phosphorus-containing organic compounds other than phytate at a slower rate [7, 9]. In contrast, phytases from *Aspergillus fumigatus* and *Klebsiella pneumoniae* showed broad specificity for phosphorylated substrates but relatively low specificity for phytate [27].

In conclusion, phytase $AppA_S$ expressed in *P. pastoris* ($rAppA_P$) had biochemical properties similar to that expressed in *E. coli* ($rAppA_E$), except for thermalstability. The enzyme has several advantageous properties, like substrate specificity, protease resistance, optimal activity at acidic pH and physiological temperature. Phytase AppA from *Shigella* sp. CD2 displayed 40–70% activity in the pH range 3.5 to 6.5, which can facilitate phytate degradation in salivary gland (pH 5.0–7.0), stomach (fed state pH 6.5, reducing to 3.5–4.5 upon stimulation of acid secretion) and upper part of duodenum (pH 4.0–6.0). Hence, the enzyme can be used as feed additive for improving the utilization of phytate phosphorus by monogastric animals like, swine, poultry and farm animals. Though production of $rAppA_P$ as secreted protein is advantageous for industry, its economical production requires improving its expression by using *P. pastoris*-preferred codons and optimization of bioprocess and scaling up when the cells are grown in a fermenter. Hence, there is a potential to increase the expression level even further, which is being pursued in the laboratory.

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Author Contributions

Conceived and designed the experiments: SG. Performed the experiments: MPR SD DM SPS. Analyzed the data: MPR SG. Contributed reagents/materials/analysis tools: SG. Wrote the paper: SG MPR.

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