“Screening of rhizobial diversity from the Wild medicinal legumes growing in Lucknow and adjoining areas”

Thesis
Submitted to

Babasaheb Bhimrao Ambedkar University
Lucknow

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For the degree of
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In
Environmental Microbiology

Submitted By
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2018

DEDICATED

TO

MY PARENTS

Certificate

This is to certify that the thesis titled **"Screening of rhizobial diversity from the Wild medicinal legumes growing in Lucknow and adjoining areas"** submitted by **Ms. Maya Verma** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations-1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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This is to certify that I have worked on the research thesis entitled **“Screening of rhizobial diversity from the Wild medicinal legumes growing in Lucknow and adjoining areas”**. The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree.

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%	Percent
° C	Degree Celsius
° E	Degree East
° N	Degree North
µg	Micro Gram
µL	Micro Liters
µS/cm	Micro-Siemens per Centimeter
ACC	1-Aminocyclopropane-1-Carboxylate
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Sequence Tool
BNF	Biological Nitrogen Fixation
bp	Base Pairs
BTB	Bromothymol Blue
CAS	Chrome Azurol S
CFU	Colony Forming Unit
cm	Centimeters
CMC	Carboxymethylcellulose
CRYEMA	Congo Red Yeast Extract Mannitol Agar Media
DAS	Days After Sowing
DMRT	Duncan’s Multiplicity Range Test
DNA	Deoxy Ribonucleic Acid
EC	Electrical Conductivity
EDS	Energy Dispersive Spectroscopy
EPS	Exopolysaccharides
FT-IR	Fourier Transmission Infra-Red
g	Gram
GAs	Gibberellins
GPA	Glucose Peptone Agar
ha	Hectare
HAM	Hofer’s Alkaline Media
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethylammonium
hrs	Hours
IAA	Indole-3-Acetic Acid
IAR	Intrinsic Antibiotic Resistance
K	Potassium
Kg	Kilogram
KNO_3	Potassium Nitrate

LPWG	Legume Phylogeny Working Group
m	Meter
mg	Milligram
Mha	Million Hectares
ml	Mili Liters
MR-VP	Methyl-Red and Voges-Proskauer
MTCC	Microbial Type Culture Collection
N	Nitrogen
N_2	Dinitrogen
$NaNO_3$	Sodium Nitrate
NBAIM	National Bureau of Agriculturally Important Microorganisms
NH_3	Ammonia
NH_4Cl	Ammonium Chloride
NH_4SO_4	Ammonium Sulphate
nm	Nano Meter
PDA	Potato Dextrose Agar
PGP	Plant Growth Promoting
PGPR	Plant Growth Promoting Rhizobacteria
PHB	Poly-P-Hydroxybutyrate
PIPES	Piperazine-N, N'-Bis (Ethane Sulfonic acid)
PSB	Phosphate Solubilizing Bacteria
PSI	Phosphate Solubilization Index
PSU	Percent Siderophore Unit
RAPD	Random Amplified Polymorphic DNA
rpm	Rotation per Minutes
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHO	World Health Organization
YEMA	Yeast Extract Mannitol Agar
Zn	Zinc
ZSI	Zinc Solubilization Index
α	Alpha
β	Beta
ϒ	Gama

INTRODUCTION

Introduction

Nitrogen (N) is one of the most important nutrients for plant growth and development (Vitousek and Howarth 1991). It is estimated that total plant dry matter contains 1–5% of N which makes essential components of plant cell such as proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones and other secondary metabolites (Hawkesford et al. 2012). Large amount of nitrogen is required for plant growth to fulfill its cellular need. N is very abundant in atmosphere in the gaseous form and over every area of land about 8,000 pounds N is found in the air. Although atmosphere contains 80% of dinitrogen (N_2), but unfortunately this is not suitable for direct plant uptake (Abd-Alla et al. 2014; Allito et al. 2015). For agricultural crop production nitrogen is essentially required and for this purpose requirement of chemical nitrogen fertilizer is increasing (Galloway et al. 2008). It is reported that to fulfill the food requirement of world large amount of nitrogenous fertilizer is required which was estimated up to more than 190.4 million ton by 2015, but this excessive use of synthetic fertilizers cause unacceptable levels of water pollution (eutrophication of water bodies) and land degradation by causing leaching or denitrification process (Dixon and Wheeler 1986; Sharifi et al. 2011). These harmful processes are economically and environmentally very unsafe (Luce et al. 2011). Hence, there is very urgent requirement of a promising alternative to these harmful chemical fertilizers. In this context symbiotic interaction of root/stem nodule inhabiting bacteria (rhizobia) and legumes is recognized as potent combination to replace mineral N-fertilizers due to their efficient capability of nitrogen fixation (Sprent 2009; Tairo and Ndakidemi 2013). This symbiotic interaction improves the nitrogen requirement and productivity of the ecosystems in beneficial way without causing any harm to the environment (Vitousek et al. 2002; Arora et al. 2017).

Rhizobia-legume symbiosis starts with two free living organisms (leguminous plants and diverse rhizobia) with very close cellular co-existence (Long 2001). In this biological interaction process rhizobial cells are transformed into bacteroids (suitable for nitrogen fixation) and fix atmospheric nitrogen into ammonia by the process of biological nitrogen fixation (BNF) (Maroti and Kondorosi 2014). It is estimated that BNF contributes in total nitrogen economy of the globe with 65% share of nitrogen resources (Sprent 2009). Rhizobia-legume symbiosis plays a crucial role in increasing productivity and quality of crops by providing approximately 40 million tonnes of nitrogen into agricultural systems each year which is equal to US $ 160-180 billion (Herridge et al. 2008; Krapp et al. 2011; Rajwar et al. 2013). The legume-rhizobia symbiosis signifies as a renewable source of N and plays important role in agriculture through intense effect on soil quality, lowering cost of agriculture, sustainability and combating climate change (Peoples et al. 1995; Lindstrom et al. 2010; Black et al. 2012).

Leguminosae is also known as Fabaceae and it is the 3rd largest family among flowering plants (angiosperms) after Asteraceae and Orchidaceae. Legumes have been recognized to be the second most economically valuable plant sources after Poaceae (Vietmeyer 1986; Arisdason and Lakshminarasimhan 2017). Leguminosae cover 12–15% of all available arable land which contributes more than 25% in the world’s primary crop production with 247 million tons of grain legumes annually (Ferguson et al. 2010). Leguminous plants are broadly defined by their unusual flower structure and podded fruit (de Faria et al. 1989). Legumes are cosmopolitan in distribution and very diverse in morphology, habitat and ecology occupying a wide range of habitats (all biomes across the globe ranging from Arctic annuals to tropical trees) and occur in even the most extreme habitats (Schrire et al. 2005). Currently 770 genera and more than 19,500 species of the legumes are reported with very large and diverse group, ranging from small plants (e.g.

clovers) to large trees (e.g. *Acacia* species) (Lewis et al. 2013; LPWG 2017). In India, legumes are represented by about 1152 species belonging to 179 genera (both indigenous and introduced) (Arisdason and Lakshminarasimhan 2017). Legumes are pioneer plants, which can provide their own nitrogen in symbiosis with root nodule bacteria and frequently planted on degraded soils, as they can improve soil fertility and also soil texture (Shamseldin et al. 2005; Stagnari et al. 2017).

Legumes are important source of various nutrients such as proteins, starch, fibre, vitamins and minerals and impart a very big role in global food security. Among all legumes pulses have large contribution and known as chief agricultural legumes. In this regard the United Nations declared 2016 as the “International Year of Pulses (grain legumes)” under the heading ‘Nutritious seeds for a sustainable future’ to spread awareness about beneficial legumes (LPWG 2017). Besides pulses there are certain other important legumes have beneficial applications for humans such as cosmetics, food, oil, fibre, fuel, timber, forages and bio-fuel. Apart from these applications legumes includes diverse potent medicinal plants with significant quantities of phenolic compounds (phenolic acids and flavonoids) having promising medicinal properties (Bhat and Karim 2009; Nikkhah 2014). Among all legumes various wild and underutilized legumes have more potential to be used as human food, animal feed and traditional medicines (Nikkah 2012; Ebert 2014; Kamboj and Nanda 2017). According to Ramakrishna et al. (2015) large amount of medicinal plants are being extracted for drug and pharmaceutical industries from wild and uncultivated habitats. Wild medicinal and underutilized legumes have higher nutraceutical values with natural biopharmaceutics for improving human and animal health (**Fig.1**) (Bhat and Karim 2009; Nikkhah 2014). In this regard various wild medicinal legumes are traditionally is in high demand all over the world to cure various diseases. India has rich collection of diverse wild medicinal legumes and used as traditional folk medicines. Some important wild medicinal legumes growing in central Uttar Pradesh (Lucknow and adjoining areas)are used in this study. *Abrus precatorius* L. (Ratti) is used in diabetes, nephritis and leucoderma (Bobbarala and Vadlapudi 2009), *Crotalaria juncea* L. (Sunn-hemp) is used as anti-inflammatory and antiulcerogenic (Ashok et al. 2006), *Clitoria ternatea* L. (Aprajita) is used in insects and snake bites (Mukharjee et al. 2008), *Leucaena leucocephala* L. (Subabool) to control stomachache, as contraception, anthelmintic, antidiabetic (Gamal-Eldeen et al. 2007), *Sesbania bispinosa* L. (Dhaincha) is used for wounds, and snakebite victims to induce emesis and cure (Kirtikar and Basu 1975) but most of these plants are neglected. Moreover these medicinal legumes also have more potent role in reclamation of waste lands thus their cultivation is recommended to improve the soil fertility. It is reported that legume diversity is declining mainly from their wild habitat due to the habitat loss (Pandey et al. 2006; Kiran et al. 2009; Bargali 2016). Neglecting wild medicinal legumes has negative impacts on human health and sustainable food production (Foyer et al. 2016). Hence, there is requirement of their conservation which can be possible by exploration and application of their symbiotic partners (rhizobia) (Singh and Sharma 2013; Arora et al. 2017). Rhizobia of wild legumes growing in arid zones exhibit higher tolerance to various environmental stresses salt stress, elevated temperature, flood and drought. Selection of efficient and compatible rhizobial strains could help in increase of plant growth and soil fertility also (Ali et al. 2009; Gopalakrishnan et al. 2014).

Rhizobia are heterogeneous group of Gram negative, aerobic, non-sporulating, rod-shaped bacteria belonging to the class α-proteobacteria and β-proteobacteria (Young and Haukka 1996; Sahgal and Johri 2003; Tak et al. 2017). Rhizobia are very diverse due to their wide geographical distribution, diverse host plants and habitats all over the globe (Martínez-Romero and Caballero-Mellado 1996). In the last few years, many studies reported the considerable phenotypic and genotypic diversity among strains, and several distinct groups have been identified which is continuously increasing with exploration of large number of legume species (Shetta et al. 2011; Berrada and Fikri-Benbrahim 2014; Huang et al. 2018). The current taxonomy of rhizobia shows their broad diversity at the genus, species, and intra-species levels having 238 nodulating species in 18 genera including *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Ensifer* (formerly *Sinorhizobium*), *Methylobacterium, Devosia, Microvirga, Ochrobactrum, Phyllobacterium, Shinella, Allorhizobium, Pararhizobium, Aminobacter, Blastobacter* and *Photorhizobium* from α-proteobacteria while *Burkholderia/Paraburkholderia* and *Cupriavidus* from β-proteobacteria subclass (**Fig. 2**) (Shamseldin et al. 2017; Rao et al. 2018). The diversity assessment of rhizobia mainly associated with less explored or unexplored plants needs more attention at global level which can be beneficial to reveal many new rhizobial species (Brunel et al. 1996; Tak et al. 2017). According to Berrada and Fikri-Benbrahim (2014) only 23% of known legumes are explored for their symbiotic associations with rhizobia hence large numbers of legumes remain unexplored. Tilak et al. (2005) and Maheshwari (2013) suggested that to find the large diversity of rhizobia selection of wild legumes and legumes growing in rare habitats are very important. Various studies also suggested that recent advancements in the taxonomic research through development of specific molecular tools and the availability of sensitive and accurate PCR-based fingerprinting methods have promising role in detection of high rhizobial diversity (Pongsilp 2012).

Legumes harbour diverse rhizobial genera with various species but some non-rhizobial genera are also reported from various studies (Selvakumar et al. 2008; Aeron et al. 2015; Martinez-Hidalgo and Hirsch 2017). Currently various non-rhizobial isolates are being explored from root nodules of various legumes and these studies showed a more complex bacterial composition including bacteria from very different phylogenetic groups which was not observed before (Rajendran et al. 2012; Velázquez et al. 2017). It is reported in some studies that these non-rhizobial isolates help in nitrogen fixation and nodule forming potential of rhizobia but it is not much proved and it require more examination.

Rhizobia are member of the plant growth promoting rhizobacteria (PGPR) group with various plant growth promoting attributes such as production of phytohormones (indole acetic acid (IAA) and gibberellin), solubilization of phosphates and zinc, production of siderophores and exo-polysaccharides (EPS) as well as biocontrol activity against phytopathogens (Arora et al. 2001; Gopalakrishnan et al. 2014; Aeron et al. 2017). Due to these PGP activities rhizobia enhance the plant growth on application as bioinoculants (Rajpoot and Panwar 2013; Laranjo et al. 2014). For preparation of efficient rhizobial inoculants, it is important to select the compatible rhizobial partner having high nitrogen-fixing efficiency for a specific leguminous crop (Herridge et al. 1994). Now use of biofertilizers is becoming more popular at the global level and amongst all types of biofertilizers, nitrogen fixers contribute maximum (75%) in agriculture (Grand View Research 2015). Rhizobial inoculants were the first biofertilizers used for the agricultural crop productions (Nobbe and Hiltner 1896). Currently, various types of rhizobial species have been discovered but only few of them are used as inoculants (Arora et al. 2017). It has been estimated that 2000 tons of rhizobial inoculants of worth US$ 50 million are produced worldwide every year (Ben Rebah et al. 2007) and this quantity is suitable to inoculate 20 million hectares of legumes (Herridge et al. 2002). The application of rhizobial bioformulations is one of the cheapest and eco-friendly approaches for improving production of leguminous plants and fixation of atmospheric nitrogen (Thakare and Rasal 2000; Mia et al. 2007; Arora et al. 2017). Rhizobia have significant importance in the field of sustainable organic agriculture.

Rhizobia are associated with legumes growing in various geographical locations and there is need of important steps for the exploration of wild plants inhabiting in non-agricultural or uncultivated areas to determine the rhizobial diversity. Since the genetic diversity of microsymbionts of the wild medicinal legumes has not been studied so far hence many of the reported important wild legumes and their associated rhizobia are getting extinct even without our knowledge. In this regard there is need to conserve the wild medicinal legumes and mining of root nodulating bacteria from these legumes (Maheshwari et al. 2013; Huang et al. 2018). This work will not only help in knowing about the rhizobial diversity of neglected wild and medicinal legumes but will also help in conservation of both the symbiotic partners which has not been done so far. In broad sense the main objective of this work is to explore the diversity (phenotypic and genotypic) of root nodulating bacteria associated with wild medicinal legumes collected from diverse uncultivated/wild sites (wastelands) and their characterization as PGPR. Application of selected rhizobial isolates to enhance the growth of respective host plants is also done.

Objectives:

1) Isolation of root nodulating bacteria from wild medicinal legumes growing in and around Lucknow region.
2) Biotyping of isolated rhizobia.
3) Molecular characterization of the rhizobial isolates and their phylogenetic analysis.
4) Check the nodulation capacity of isolated strains for the host and diverse legumes.
5) In vitro and in vivo studies to check the impact on growth by the isolated root nodulating bacteria for their respective host.
6) Submission of useful strains in culture collection centres.

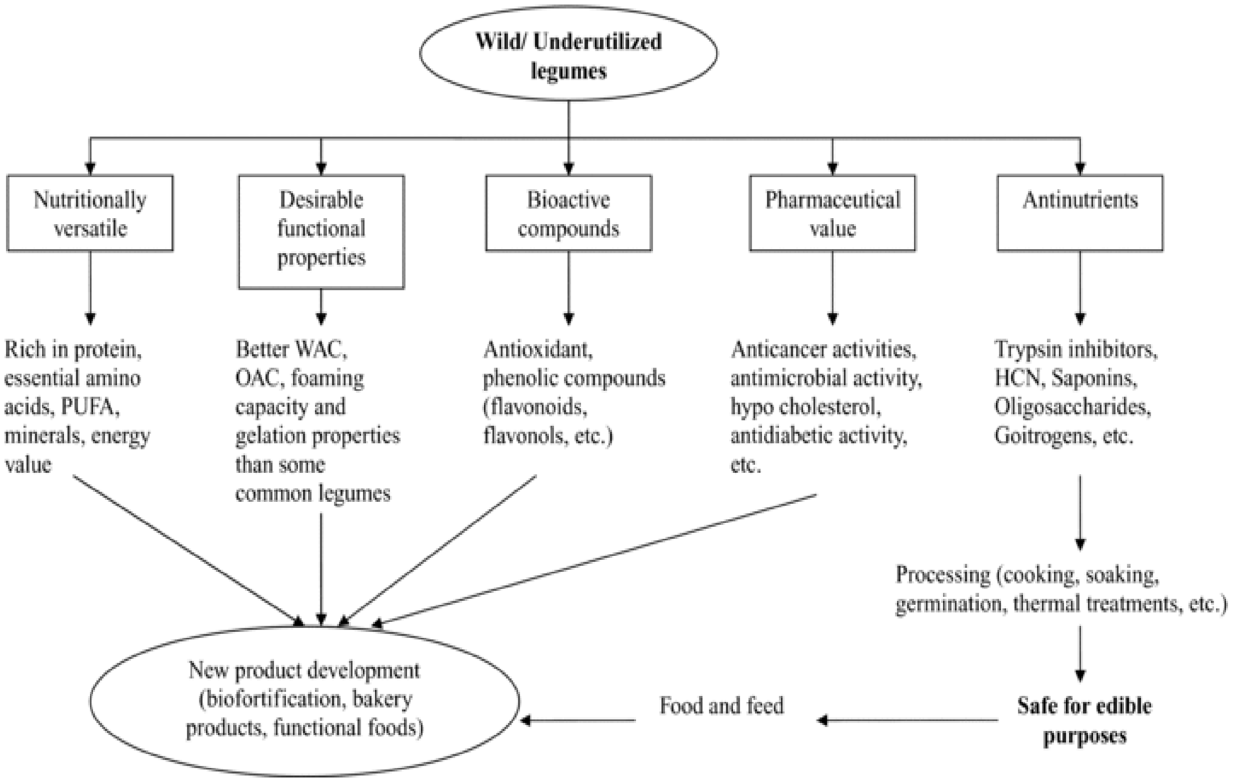


Fig. 1: Importance of wild medicinal legumes in various fields (Bhat and Karim 2009)

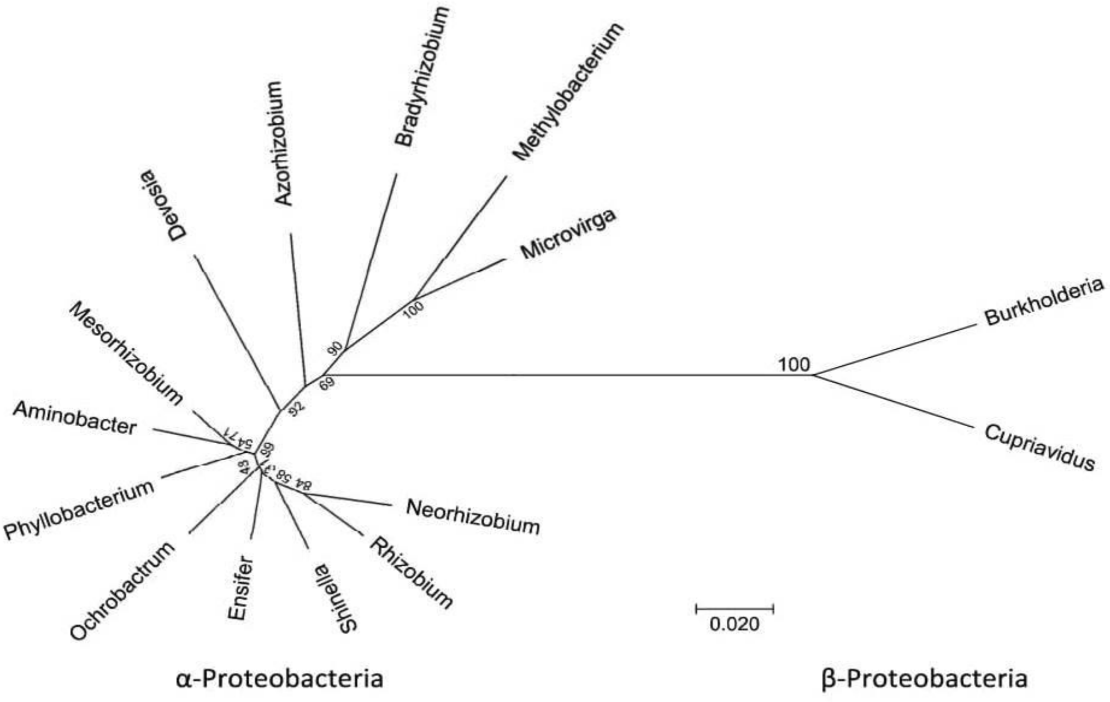


Fig. 2: Phylogenetic tree showing diverse rhizobial genera (Tak et al. 2017)

REVIEW
OF
LITERATURE

Nitrogen is the most vital nutrient for plant growth and a basic requirement for life on earth. Nitrogen is the main component of proteins, enzymes, chlorophyll and growth regulators of plants and its scarcity causes limited growth, leaf yellowing, reduced branching and small trifoliate leaves in plants (Ougham et al. 2008). Nitrogen present in the environment is not directly available for plant uptake. The fixed form of N is generated by conversion of N_2 to NH_3, a process also known as nitrogen fixation. Generally, the most reduced form of N (ammonia) or most oxidized form (nitrate) are needed for plant uptake. Plant growth and development is controlled by various activities and nitrogen fixation is the second most important process after photosynthesis which affects primary productivity of plants. Due to high requirement of food for increasing population of world high crop productivity is needed for food security, hence huge amount of synthetic nitrogen (N) is applied in agriculture. It is estimated that in the last 40 years application of N fertilizers has increased from 12 Tg/year to 104 Tg/year (McAllister et al. 2012). It is reported that chemical nitrogenous fertilizers are popularly applied for cereals and other agricultural crops since the Second World War (Davidson 2009). This injudicious application of chemical fertilizers causes various side effects in the environment mainly water pollution, eutrophication and soil degradation (Carpenter et al. 1998). Due to these consequences sustainable approach of nitrogen fixation is needed for higher crop productivity in eco-friendly manner.

Nitrogen fixation occurs by two main processes, biological or non-biological. Non-biological fixation includes geochemical fixation by lightning which is 10% of the total N_2 fixation and industrial fixation by Haber-Bosch process which is 15% of total N_2 fixation (**Fig. 3**) (Bezdicek and Kennedy 1998). Biological nitrogen fixation is carried out by various microorganisms, including a small but diverse group of bacteria and archea. These microbes are commonly known as diazotrophs (Zehr et al. 2003; Kneip et al. 2007). It is estimated that over half of the fixed N_2 is supplied biologically and has an intense agronomic, economic, and ecological impact (Smil 2001). Microbes responsible for nitrogen fixation are divided into two categories: (a) symbiotic N_2 fixing bacteria including members of the family Rhizobiaceae which forms symbiosis with leguminous plants (e.g. Rhizobia) (Ahemad and Khan 2012) and non-leguminous trees (e.g. *Frankia*) and (b) non-symbiotic (free living, associative and endophytic) nitrogen fixing forms such as Cyanobacteria (*Anabaena*, *Nostoc*), *Azospirillum*, *Azotobacter*, *Gluconoacetobacter diazotrophicus* and *Azocarus* etc. (**Fig. 4**) (Bhattacharyya and Jha 2012). These microbes are able to fix atmospheric nitrogen into soil and make plant utilizable forms (NH_3) by using the nitrogenase enzyme complex (Kiers et al. 2003). BNF contributes about 100 million tons of nitrogen for terrestrial ecosystems and 30 to 300 million tons for marine ecosystems (Rakash and Rana 2013). BNF is an efficient source of fixed N, which plays an important role in land remediation and pollution reduction.

Due to high requirement of BNF symbiotic association between legumes and rhizobia is highly concerned. Rhizobia-legume symbiosis has the greatest quantitative impact on the nitrogen cycle and represents a renewable source of N for agriculture. This emphasizes the consequence of rhizobia–legume symbioses as a major contributor to BNF (Zahran 1999). Rhizobia-legume symbiosis is the major contributor of biologically fixed nitrogen in comparison to non-symbiotic nitrogen-fixing bacteria (Venieraki et al. 2011). This system is estimated to contribute 1.44×10^8 metric tons of nitrogen per year at global level (Gothwal et al. 2007). The legume-rhizobia symbiosis has very significant impact on success of leguminous crops (Kahindi et al. 1997). It is reported that rhizobia inhabiting root nodules of legumes reduce approximately 20 million tons of atmospheric nitrogen to ammonia which is 50%-70% of the total BNF around the globe (Graham and Vance 2003). The higher fixed nitrogen in hosts determines the success of symbiotic relationship between legumes and rhizobia (Crews 1999). Therefore, legume-rhizobia symbiosis can provide an easy and inexpensive way to enhance soil fertility and improve crop production without causing any harm to the environment (Roychowdhury et al. 2013).

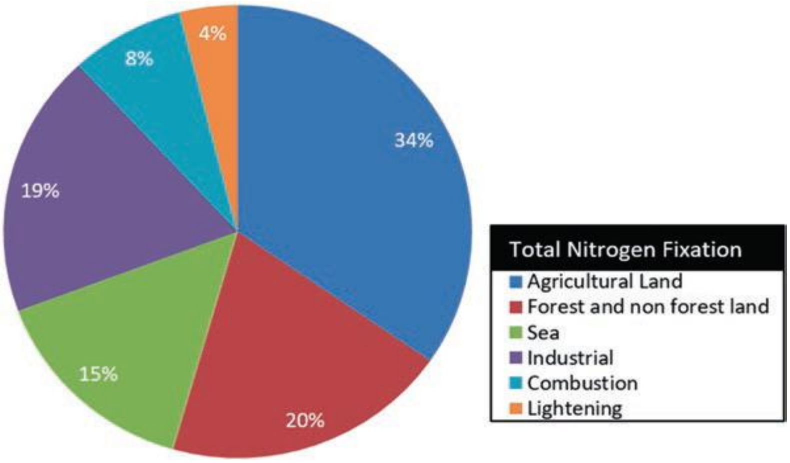


Fig. 3: Nitrogen fixation on earth (Modified from Bezdicek and Kennedy 1998)

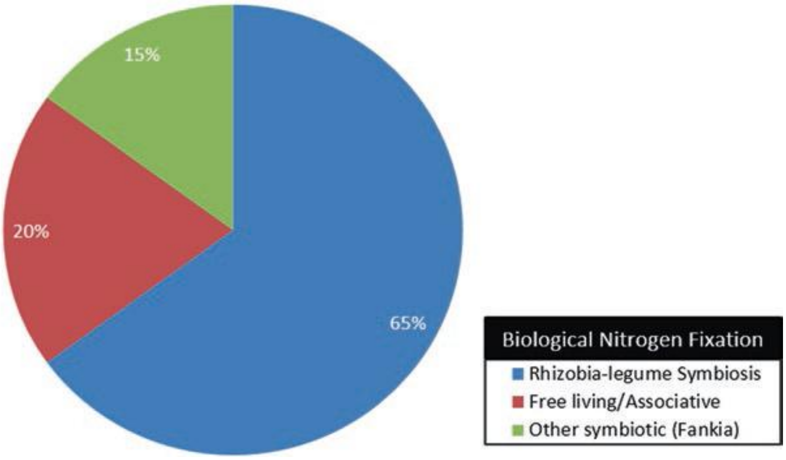


Fig. 4: Biological nitrogen fixation (Modified from Bouizgarne et al. 2015)

2.1 *Leguminosae*

Legumes are very diverse group of plants on earth and widely distributed in both tropical and temperate regions of world (Sprent and James 2007). Legumes have been domesticated alongside grasses in different areas of the world since the beginning of agriculture and have potent role in its early development (Hancock 2012). Legumes belong to the angiosperm family of plant named Leguminosae or Fabaceae of order Fabales, in eurosid clade (Tran and Naguyen 2009). Legumes are the main components of major vegetation of the world (Sprent 2009). These plants are ubiquitous with very diverse nature such as large emergent tropical trees with buttresses, small ephemeral annual herbs, climbing annuals or perennials with tendrils, desert shrubs, geoxylic subshrubs, woody lianas and, less commonly, aquatics (LPWG 2013). They are abundant in the lowland wet tropical forests in Africa, South America, and Asia and dominantly grow in dry forests and savannas throughout the tropics, and also found in Mediterranean, desert and temperate regions, up to high latitudes and at high elevations (DRYFLOR 2016). The legume family is divided into three sub-families, the Caesalpinioideae, Mimosoideae and Papilionoideae (Lewis et al. 2005). It is reported that large numbers of legume species have nodulation as generic character and 96% member of subfamilies Papilionoideae and Mimosoideae show nodulation while only few members of the subfamily Caesalpinioideae (22%) show nodulation property (Sprent 2009). Recently Legume Phylogeny Working Group (LPWG) published the new classification of family Leguminosae which is based on comprehensive phylogeny of legumes. According to this classification family Leguminosae is divided into 6 new subfamilies Caesalpinioideae (incl. mimosoid clade), Dialioideae, Detarioideae, Cercidoideae, Duparquetioideae and Papilionoideae (**Table 1**) (LPWG 2017).

Table 1: New subfamily classification of the *Leguminosae* (LPWG 2017)

S. No.	Subfamily	Genera	Species
1.	Caesalpinioideae (incl. mimosoid clade)	146/148, 937/ca.	4400
2.	Dialioideae	15/17, 19/ca.	85
3.	Detarioideae	79/84, 327/ca.	760
4.	Cercidoideae	12/12, 96/ca.	335
5.	Duparquetioideae	1/1	1/1
6.	Papilionoideae	445/503, 2316/ca.	14,000

Leguminosae has many economically and ecologically important roles and described as very important family among all the dicotyledonous plants (Harborne 1994). Legumes are important source of food crops and provide essential nutrients such as protein and micronutrients that can affect human and animal health (Yahara et al. 2013). Legumes are grown for production of food, oil, fiber, fuel, timber, medicines, forages, biodiesel fuel and chemicals. Despite its major role in food requirements of the world, legumes offer multiple benefits to both soil and other crops through intercropping (Stajković et al. 2011). Some popular grain legumes crops such as soybean, cowpea, common bean, chickpea and peas are important source of human food, animal feed. They can be grown in different agro-ecological zones and work as soil fertility enhancers (Hendawey and Younes 2013). Legume-rich diet has various beneficial health effects on humans and livestock. Hence, global food security and soil fertility could be significantly improved by higher grain legume usage. Legumes are also uniquely important as fodder and green manure all over the world and also used for their wood, tannins, oils and resins, in the manufacture of varnishes, paints, dyes and medicines, and in the horticultural applications (LPWG 2017). Legumes are also used as traditional and folk medicines in various countries of the world (Ebert 2014). Despite various advantages some legumes of immense medicinal and other beneficial values are underutilized resulting in compromised human health, food security and soil sustainability (Foyer et al. 2016). Legumes growth and productivity is controlled by fixed nitrogen in the soil with suitable climatic conditions (Agren 1985). Legumes can easily survive even in poor soils with less nitrogen content due to their symbiotic association with rhizobia (Freitas et al. 2004). Leguminous plants reduce the environmental pollution due to their symbiotic atmospheric nitrogen fixation activity, which affords economically sustainable advantages for agriculture (Gothwal et al. 2007). This family is morphologically, physiologically and ecologically very diverse and represent one of the most remarkable examples of evolutionary diversification in plants. Legumes are important crop and their successful distribution in world critically depends on the availability of compatible rhizobial associations (Mutch and Young 2004).

2.2 Wild medicinal legumes:

Medicinal plants are very important source of herbal medicines all over the world since ancient time. These plants are used in different folk and traditional medicinal systems such as Ayurveda, Unani and Siddha. According to world health organization (WHO) more than 75% of the world's population use traditional herbal medicines for their health care need (WHO 2004). WHO also estimated that the global market for traditional medicinal products can reach upto US $ 5 trillion by 2050 (Subrat 2005). This indicates the tremendous potential and demands in this sector. Medicinal plants are rich source of certain chemicals (secondary metabolites) and potentially useful to produce various types of herbal medicines that are used in treating and preventing specific ailments and diseases. These chemicals can be extracted for the manufacture of various pharmaceutical products for medical porposes. Due to great demand of medicinal plants, currently there is much need to cultivate them on higher scale to fulfil the high demand of biologically active compounds used by food, pharmaceutical and health care industries (Chen et al. 2016). International Union for Conservation of Nature (IUCN) and the World Wildlife Fund (WWF) estimated that there are between 50,000 and 80,000 flowering plant species used for medicinal purposes at global level (Chen et al. 2016). Among these, about 15,000 species are threatened with extinction due to over exploitation and habitat destruction (Bentley 2010). It is reported that 20 % of their wild resources have already been exhausted with the increasing human population and plant consumption (Ross 2005). However, herbal medicines are used all over the world but most countries of Asia are capable of acquiring medicinal plants for their traditional uses, very few are capable of producing them at commercial (Chapman and Chomchalow 2005).

India is one of the largest producers of herbs and herbal products. India has one of the richest plant medical cultures in the world and these plants are known as rich heritage of our country. India is enriched with the world's 12 richest biodiversity, cultural and traditional system in respect to medicinal plants. India has 16 agro-climatic zones with 45,000 species of plants and among them 15,000-20,000 have potential medicinal values (Gaur and Kaushik 2011). India harbours about 3000-3500 medicinal plants which is 15% of total medicinal plants of the world (Bhuvaneswari et al. 2015). In India the oldest record of the use of medicinal plants is given in the Rig-Veda (4500-1600 BC) and Charaka Samhita (1000-800 BC) and Susruta Samihta (800-700 BC) are the two important systems of medicine. Medicinal plants play an essential role in the primary healthcare and formed the basis of traditional medicine systems. Apart from medicinal values cultivation of medicinal plants also has many advantages such as helping to conserve endangered species in their natural habitat, permitting production of uniform materials, providing good income to the farmers, providing opportunities for value-addition through processing, providing a better environment through utilizing waste and unproductive lands, and providing continuity of supply (Chapman and Chomchalow 2005). Many of the leguminous plants have diverse medicinal values and used as folk medicines to treat various human diseases since ancient times (Ebert 2014). In the last few years the study of other legume components whose interest is more related with the human health gained interest (Polak et al. 2015). In addition of high quality proteins, legumes contain several bioactive compounds such as polysaccharides, bioactive peptides, phenolics, including isoflavones, carotenoids, tocopherols and fatty acids, amongst other phytochemicals, which make them as excellent functional foods and nutraceutical products (Bhat and Karim 2009).

Medicinal plants are found in all the ecological and geographical sites but wild type of plants has more medicinal values. It has been estimated that four out of five medicinal plants utilized by man are collected from the wild (Srivastava et al. 1995) and out of 20, 000 medicinal plants of the world, about 90 % of these are found growing wild in different climatic regions globally (Bhuvaneswari et al. 2015). Wild species harbour many useful traits and have potential to thrive under extreme climatic conditions. In this context very recently scientists report significant steps in transferring disease- and stress-resistance traits from wild legumes to their domesticated varieties through pre-breeding by using wild species for genetic enhancement of grain legumes (Sharma 2017). There is need of concern for conservation of medicinal plants because of threat of extinction due to forest destruction, over cropping, overgrazing and desertification (land degradation) (Chapman and Chomchalow 2005). Wild legumes are widely distributed in arid regions of the world and actively enhance the soil fertility in those environments due to nitrogen fixing activity (Zahran 2001). Wild legumes have more tolerance to adverse environmental conditions with higher nitrogen fixing activity in comparison to crop legumes and have more potential for reforestation and land conservation (Ahmad et al. 1984). Mining of wild and underutilized legumes is significant for food security and agricultural development and thus can effectively enhance the national economy of the country (Arinathan et al. 2003). Many wild and underutilized legumes have sufficient amounts of various bioactive compounds such as protein, essential amino acids, polyunsaturated fatty acids (PUFAs), dietary fiber, essential minerals and vitamins than other common legumes (Awoyinka et al. 2016; Silva et al. 2017). Various members of wild medicinal legumes are used for medical purposes and medicinal values of some selected wild medicinal legumes are included in the present study (**Table 2**) are discussed below:

Table 2: Detail of selected wild medicinal legumes

S. No.	Name of plants	Subfamily	Medicinal value	References	Pictures of plants
1.	*Abrus* (Ratti)	Papilionoideae	Used in diabetes, nephritis and leucoderma Used as neuro-protective, anti-viral, antifertility, nephroprotective and immunomodulator	Bobbarala and Vadlapudi (2009); Garaniya and Bapodra (2014)	
2.	*Clitoria* (Aprajita)	Papilionoideae	used in insects and snake bite Anti-inflammatory antipyretic and analgesic effects Antiparasitic and insecticidal effects	Mukharjee et al. (2008)	
3.	*Crotalaria* (Hemp)	Faboideae	used as anti-inflammatory and antiulcerogenic used in traditional medicine to treat urinary problems used to treat painful swelling of joints	Ashok et al. (2006)	

4.	*Leucaena* (Subabul)	Mimosoid clade of the Caesalpinioideae	It is used to control stomachache and also as anthelmintic and antidiabetic agent It also has a broad spectrum antibacterial activity	Gamal-Eldeen et al. (2007)	
5.	*Sesbania* (Dhaincha)	Faboideae	It is used as antidiabetic and anti-diaorrheal it is used for wounds, and snakebite victims to induce emesis and cure	Misra and Siddiqi (2004); Kirtikar and Basu (1975)	

2.2.1 *Abrus* sp.

A. precatorius L. is a high-climbing, twiner/trailing woody vine with slender herbaceous branches and commonly known as Ratti, Gunja or Jequirity. It mainly grows in dry regions at lower elevations, especially in the tropical and subtropical parts of the world. Although it is native plant of India but also grows in other countries of the world such as South Africa, China, Islands, West Indies, Brazil etc. In India it is found in all geographical regions throughout the plains, from Himalaya down to Southern India (Kirtikar and Basu 2005). *Abrus* is used as a multipurpose medicinal legume and have many potent pharmacognostical and pharmacological characters with medical applicability (Garaniya and Bapodra 2014). This plant also has many important vitamins, minerals, amino acids and carbohydrate (Arora et al. 2011). It has many important phytochemicals including flavonoids, glycosides, abrin and alkaloids which are responsible for its medicinal properties (Alayande et al. 2017). It is used as neuro-protective, abortifacient, anti-viral, antidiabetic, anti-malarial, antifertility, nephroprotective and immunomodulator (Garaniya and Bapodra 2014). All plant parts of *Abrus* has medicinal value and dried seeds are used to treat diabetes and chronic nephritis (Monago and Alumanah 2005) and also as an oral contraceptive (Watt and Breyer-Brandwijk 1962). *Abrus* also has antimicrobial, cytotoxicity and carbohydrate metabolizing enzymes with inhibitory potentials (Alayande et al. 2017). *A. precatorius* is medically very important but not much explored for their rhizobia. Ogasawara et al. (2003) reported a root nodulating bacteria *Sinorhizobium abri* from this plant.

2.2.2. *Clitoria* sp.

C. ternatea L. is a twining evergreen flowering plant and commonly known as Aparajita or Butterfly pea or blue pea. It is perennial climber with slender downy stem, found throughout the tropical regions of the world (Kosai et al. 2015). It is probably originated in tropical Asia and widely distributed throughout the humid, lowland tropics of Africa, Asia and Central America (Gomez and Kalamani 2003). It is a nutritive multipurpose forage legume for the tropics and also used as the food dyes in Nasi kerabu (the local dish in Kelantan, Malaysia). In the traditional system of medicine, ‘Aparajita’ is considered as a ‘Medhya rasayan (brain tonic)’ drug to improve intelligence and enhance memory function (Ramaswamy et al. 2011). This plant contain tannins, phlobatannin, carbohydrates, saponins, triterpenoids, phenols, flavanoids, flavonol glycosides, proteins, alkaloids, antharaquinone, anthocyanins, cardiac glycosides, Stigmast-4-ene-3,6-dione, volatile oils and steroids (Bobbarala and Vadlapudi 2009). Gollen et al. (2018) also reported various primary and secondary plant metabolites such as aparajitin, clitorin, triterpenoides, anthocyanins, steroids and flavonol glycosides from *C. ternatea* L. This plant has many pharmacological activities such as antimicrobial, antioxidant, anticancer, hypolipidemic, cardiovascular, central nervous, respiratory, immunological, anti-inflammatory, analgesic antipyretic, insecticidal and many other pharmacological effects (Mukherjee et al. 2008; Lijon et al. 2017). It is also used in the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumours (Ramaswamy et al. 2011). Juice and flowers are used as an antidote for snake bite (Ramaswamy et al. 2011). Methanol extract of flower shows anticancer activity against multiple cancer cell lines of humans such as breast carcinoma, breast adenocarcinoma, hepatocellular carcinoma, leukemia cells and anaplastic thyroid cancer (Shivaprakash et al. 2015). Rhizobial association of *C. ternatea* is reported in some earlier studies such as Ahmad et al. (1984) (*Rhizobium* sp.), Roy and Basu (1992) (*Rhizobium* sp.), Lopez-Lopez et al. (2012) (*R. mesoamericanum*) and Ghosh et al. (2006) (*Mesorhizobium thiogangeticum* from rhizosphere) but need more concern.

2.2.3 *Crotalaria* sp.

The genus *Crotalaria* contains approximately 600 species that grow wild in tropical and subtropical areas (Polhill 1981). *C. juncea* (sunn hemp) is a fast growing short-day, erect shrubby annual plant, generally 1 to 4 m in height (Polhil 2011). It is generally considered to be native to Indian subcontinent (Bangladesh, Bhutan and India) (Wiersema et al. 1990) and widely cultivated in the drier areas of the tropics and subtropics and in many temperate areas with a hot summer (Mosjidis 2006). It often escapes from cultivation, naturalizes easily and grows in many areas as a ruderal plant (Maroyi 2011). It has various agricultural, economic and pharmaceutical importances but not much exploited and considered as underutilized crop (Rodrigues 2009). *C. juncea* has carbohydrates, steroids, triterpenes, phenolics, flavonoids, alkaloids, aminoacids, saponins, glycosides, tannins and volatile oils (Dinakaran et al. 2011). The plant possessed hypolipidemic, reproductive, antioxidant, antibacterial, antifungal, anti-diarrhoeal, anti-inflammatory, hepatoprotective, and many other pharmacological effects (Al-Snafi 2016). It is traditionally used as blood purifier, abortificient, astringent, demulcent, emetic, purgative, in the treatment of anaemia, impetigo, menorrhagia and psoriasis (Bhatt et al. 2009; Dinakaran et al. 2011). Another member of *Crotalaria* named *C. pallida* show strong antibacterial activity (Kiruthiga et al. 2014). *C. juncea* is medicinally very important but it is an underutilized crop and it’s rhizobia is reported in very few studies such as Sridevi et al. (2008) (*Rhizobium* sp.), Oliveira et al. (2013) (*R. galeagae* and *R. tropici*).

2.2.4 *Leucaena* sp.

Leucaena leucocephala L. is a medium sized fast growing tree. It is commonly known as wild tamarind, kubabul or subabul (Chandrasekhara et al. 1984). It is as a long-lived and highly nutritious forage tree and cover 2-5 million ha are of the world (Awe et al. 2013). *L.*

leucocephala is widely grown in the tropics and subtropics and highly tolerant to various abiotic and biotic stresses. It can be grown under unirrigated conditions in areas with relatively warm climates such as Australia, Southern India, Africa, Central and South America, Philippines, and Taiwan (Brewbaker et al. 1985). It is successfully used as a multipurpose tree suitable for agroforestry and known as “miracle tree” (Gutteridge et al. 1998). It is also known as a "conflict tree" because it has been promoted for its forage production and naturally spreads like a weed. The kernel of seeds contains more than 20% oil and leaves and seeds also contain lipids, crude protein and carbohydrates which make it bio energy crop (Devi et al. 2013). The seed gum used as a binder in tablet formulation (Verma and Balkishen 2007). This plant has various secondary metabolites as alkaloid, cardiac glycosides, tannins, flavonoids, saponins and Glycosides (Zayed et al. 2017). It has multipurpose uses including beneficial pharmacological properties. The extracts of the seeds has reported as anthelmintic, antidiabetic, anticancer activity and also has a broad spectrum antibacterial activity (Syamsudin et al. 2010). Medicinally it is used for its antimicrobial, anthelmintic, antibacterial, anti-proliferative and antidiabetic, anticancer, cancer preventive, diuretic, anti-inflammatory, antioxidant; antitumor, antihistaminic, nematicide, pesticide, anti-androgenic, hypo-cholesterolemic, and hepatoprotective properties (Zayed and Benedict 2016). *L. leucocephala* is explored for their rhizobial association in various countries of the world but very less in India. Rhizobial species reported from this plant are *M. plurifarium* (De Lajudie et al. 1998), *Ensifer morelense* (Wang et al. 2002). *R. leucaenae* (Ribeiro et al. 2012), *R. grahamii* (Lopez-Lopez et al. 2012), *Cupriavidus necator* (Da Silva et al. 2012). Chen et al. (2013) reported *Rhiozbium, Mesorhizobium, Bradyrhizobium* and *Ensifer* from root nodules of *L. leucocephala* in China.

2.2.5 *Sesbania* sp.

Sesbania bispinosa L. is a small tree with multipurpose applications. It is native to Asia and North Africa and most commonly found in tropical Africa where it grows as a common noxious weed, and has been introduced to the America. *Sesbania bispinosa* L. is wild shrub and cultivated throughout India. It is traditionally used as folk medicines in the Ayurveda and different plant parts are used as ailments which treat all types of fever. Its leaves are good source of protein supplementation with high quality nutrients. The leaves of the plant have high concentration of (+) pinitol as antidiabetic agent and various parts of the plant has many phyto-constituents such as; flavonols, tannins, saponins, phytosterols, anthocyanins, alpha-ketoglutaric acid, oxaloacetic acid and pyruvic acid (Kumar et al. 2014). Roots are used as contraceptive, hepato-protective, anti-helminthic and carminative. Root and bark are used as bitter tonic, nervous disorders, as central nervous system stimulant (Arif et al. 2013). Barks are also used to treat the ulcer, leucorrhoea, vitiated conditions of pitta, anaemia, bronchitis, tumor, dysentery, inflammations, cirrhosis of liver and hypertension. Seeds are emmenagogue, stimulant, astringent and also used in treatment of diarrhoea, reduction of spleen enlargement and skin disease (Ramdas et al. 2010). Various species of *Sesbania* (*S. cannabina, S. herbacea, S. rostrata* and *S. virgata*) are studied for their rhizobial association from root nodules in the world but nodule symbionts of *S. bispinosa* in India are not very well reported (Shamseldin et al. 2017). Rhizobia reported from *Sesbania* are *R. huautlense, E. saheli, Azorhizobium caulinodans, A. doebereinerae. E. indiaense, E. sesbaniae, E. morelense* and *M. plurifarium* (Shamseldin et al. 2017). Recently Yan et al. (2017) and Li et al. (2016) reported *Agrobacterium salinitolerans* and E. alkalisoli from *S. cannabina* in China.

2.3 Nodule microsymbionts: Root nodulating bacteria

Root nodules are unique and highly organized structures which are developed by the symbiotic relationship between leguminous plants and bacterial group of the genus rhizobia (Gage 2004). Nodule formation integrates several developmental processes, such as induction of cortical and pericycle cell division and rhizobial invasion, which are coordinated in time and space (**Fig. 5**). When nitrogen in the soil is inadequate, legumes release flavonoids which signal to rhizobia that the plant is seeking symbiotic bacteria (Ndakidemi and Dakora 2003). In response, the rhizobia releases nodulation factor which stimulates the plant to create deformed root hairs (Banfalvi and Kondorosi 1989). Rhizobia then form an infection thread for allowing them to enter the root cells through root hairs (Gage et al. 1996). When the rhizobia are inside the root cells, the cells divide rapidly to form nodule (Dudley et al. 1987). The rhizobia enter through ‘crack entry’ or through the root hairs. The infection threads in the form of tubular structures allow the invasion of the root hairs and the underlying cortical cells by the rhizobia. Once bacteria enter into plant roots, they are transformed into bacteroids and carry out the nitrogenase-mediated nitrogen fixation (Oke and Long 1999). The nitrogenase produced by the bacteroids is oxygen sensitive and therefore need a micro-aerophilic environment to be active (Ott et al. 2005).

Legume nodules harbour two types of bacteria, the rhizobia, responsible for their formation, and other endophytic bacteria whose role in the nodule is still poorly known (Xu et al. 2014; Boukhatem et al. 2016). These bacteria constitute the nodule microbiome from which the rhizobia have been widely studied since decades, whereas the nodule endophytes have been studied only since last few years. These studies showed a more complex bacterial composition than previously thought, including bacteria from very different phylogenetic groups (Velázquez et al. 2017). Zakhia et al. (2004) proposed the term BNL (Bacteria Nodulating Legumes) to avoid confusion between the general term of *Rhizobium* and the genus name. Taxonomists have scratched their heads many times when classifying and reclassifying root-nodule-inhabiting bacteria. Continually evolving methods of molecular biology, including a polyphasic approach, have led to a record number of increases in the number of root-nodulating microsymbionts (Aeron et al. 2015).

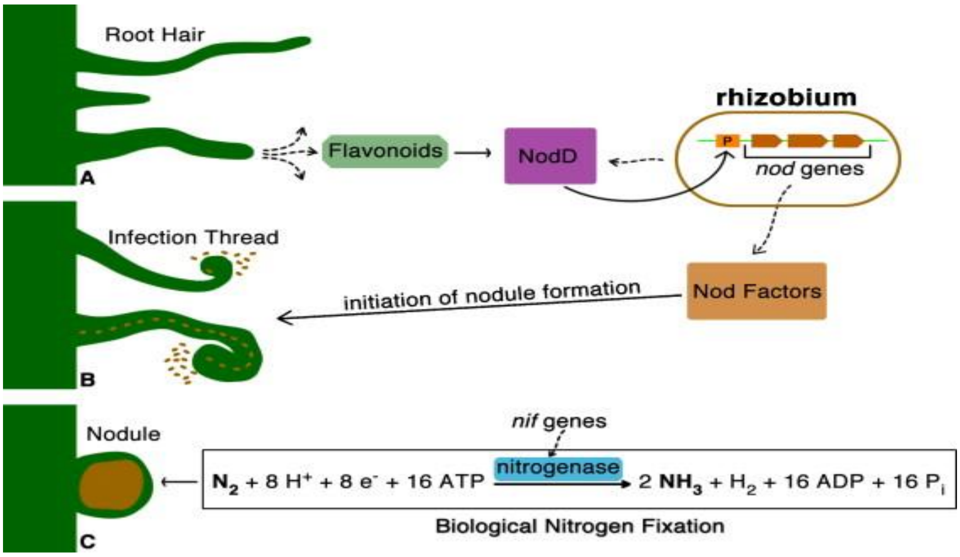


Fig. 5: Nodule formation and biological nitrogen fixation (Laranjo et al. 2014)

2.4 Rhizobia

Rhizobia are a heterogeneous group of Gram negative, non-sporulating, aerobic, motile rods with the ability to induce nodules in stems and roots of legumes (Rivas et al. 2009). In root nodules they are able to fix atmospheric nitrogen through their transformation into bacteroids (symbiotic nitrogen fixing forms) (Zahran 1999). Rhizobia are mainly considered as the diverse group of mutualistic endosymbionts (Poole et al. 2018). Rhizobia live in the environment in two different lifestyles such as free-living soil saprophytes or nitrogen-fixing endo-symbionts of leguminous plants (Shamseldin et al. 2008). Rhizobia is mainly a collection of all the nodule forming and nitrogen fixing bacteria in association with legumes and that belong to genus that were at one time part of the genus *Rhizobium* or closely related to it (Shamseldin et al. 2017). Mainly rhizobia are a generic name for a wide range of bacteria mostly fixing nitrogen in symbiosis with legumes as their host plants (Rivas et al. 2009). According to Simon et al. (2014) rhizobia is the general name given to a phylogenetically diverse group of soil bacteria that form nitrogen fixing symbioses with leguminous plants. These bacteria are environmental heterotrophs with great metabolic plasticity that may survive in many different environments, sometimes un-associated with plant hosts but have significant interest because of their ability to form symbiotic associations with legumes and are able to elicit symbiotic association on the species of Leguminosae, forming nodules in roots or stems (Rivas et al. 2009). Rhizobia are currently distributed in several families and genera (Rao et al. 2018).

2.5 History of rhiozbia

Legumes have been used as an important food source for humans and animals since ancient times. Legumes also have been used as soil quality improvers through BNF due to their symbiotic association with rhizobia in their root nodules. These symbiotic interactions have developed approximately 58 million years ago. It is reported that rhizobia have co-evolved within legumes and these legumes themselves originated about 60 million years ago (Heckman et al. 2001). However rhizobia-legume symbiosis is known as very popular interaction but its history is too old and many milestone discoveries have been done related with it (**Fig. 6**). In 1542, A German botanist Leonhard Fuchsius, first time published the drawings of nodulated legumes (Fuchsius 1542). Malpighi (1679) also observed the nodules on the root of *Phaseolus vulgaris* and *Vicia faba*. In 1838, Boussingault first time proposed BNF by legumes when he was doing crop rotation experiments and found increased N content in legumes with benefits to the soil also. Lachmann (1858) done the microscopic study of nodules and observed that nodules contain vibrio like particles. Further, these particles were also described as bacteria like and Woronin (1866) confirmed that root nodules in legumes were formed by a specific group of bacteria but Frank (1879) examined nodules and thought the symbiotic microorganism was a fungus and gave the name *Schinzia leguminosarum*. In 1886, German scientists Hellriegel and Wilfarth discovered that root nodule bacteria have ability to fix gaseous N, which was a very major finding and two years later, they published their observations (Hellriegel and Wilfarth 1888). In the same year Dutch microbiologist Beijerinck, first time isolated a bacterium from root nodules and named it as *Bacillus radicicola* (Beijerinck 1888) and later Frank renamed it as *Rhizobium leguminosarum* (Frank 1889) which has not been changed. Marshall Ward (1887) (USA) showed that root nodules are formed only in the presence of soil bacteria. After the discovery of rhizobia as nitrogen fixers Nobbe and Hiltner (1896) first time used them as “N biofertilizer” which was commercialized and patended as ‘Nitragin’. In this context Famous agricultural chemist Guthrie (1896) stated about rhizobial inoculants “one of the most valuable contributions ever made by science to practical agriculture”. After that members of the rhizobial group increased and need to classify them. Taxonomically, this particular group of bacteria is in a state of flux and their systematics present major challenges.

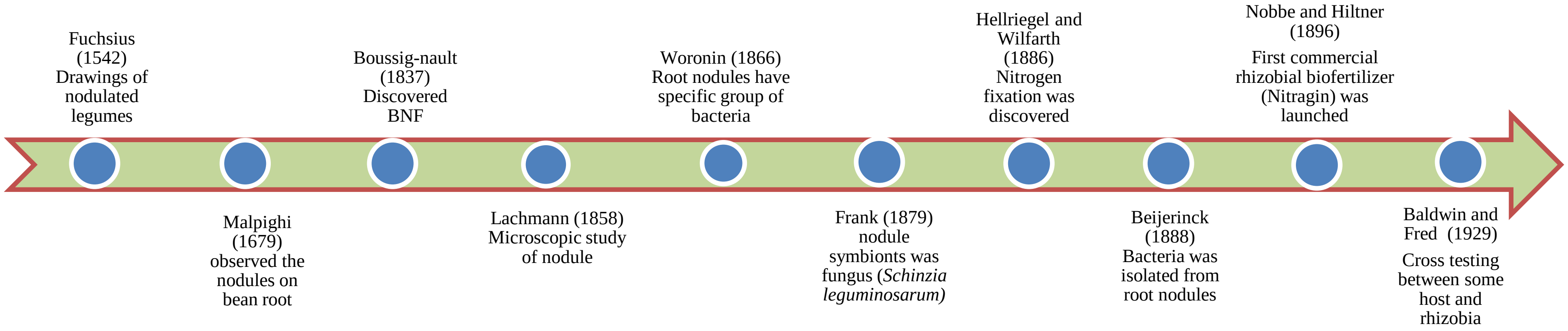


Fig. 6: History of rhizobia (Modified from Arora et al. 2017)

2.6 Classification of rhizobia

After the discovery number of rhizobial genera is continuously increasing with diverse nature hence their classification is needed. On the basis of their generation time and growth patterns in culture medium Löhis and Hansen (1921) classified the rhizobia into two major groups i) slow growers and ii) fast growers. This classification is not suitable for strain level identification; this defines only growth pattern of rhizobial starins. In 1929, Baldwin and Fred proposed the first classifications of rhizobia based on cross inoculation concept for taxonomic characterization of rhizobia. On the basis of cross inoculation between some leguminous host plants and their associated rhizobial strains rhizobia were divided in different groups. This classification was published by Fred et al. (1932) in their classic and comprehensive book on legume nodule bacteria. After this categorization not any major steps were taken for rhizobial classification about 50 years and no further changes in the nomenclature were made. However this cross inoculation concept was very popular but it was challenged by researchers who found many results of overlapping host ranges (Wilson 1944).

Classification based on phenotypic characters of rhizobia has been used as classical method of their classification and this is known as the first method of classifying unknown strains of rhizobia. This classification was performed to study the bacterial diversity and taxonomy on the basis of their cultural and morphological characters such as shape, size, surface characteristics and pigmentation, cell wall staining, salt and pH range, temperature tolerance, growth rate, pathogenic/symbiotic characters, serological relatedness, intrinsic antibiotic resistance, and bacteriophage resistance (Graham 1963; Vincent and Humphrey 1970). In the Bergey's Manual of Determinative Bacteriology (1974) six species were included in the genus *Rhizobium* (Jordon and Allen 1974). The Bergey's Manual was fundamentally important for rhizobial taxonomy in which history of the rhizobial species was recorded from the beginning of the Bacteriology until 1980 (Skerman et al. 1980). However this classification is also used today as classical method, it was changed in 1984 and continuously evolves with regular efforts. In the first edition of Bergey's Manual of Systematic Bacteriology (1984) rhizobia was divided in to two genera *Bradyrhizobium* and *Rhizobium* on the basis of their growth rate and symbiotic host ranges (Kreig and Holt 1984). Woese et al. (1984) reported the modifications in the taxonomy of prokaryotes on basis of their molecular characterization (16S rRNA gene sequencing and phylogenetic analysis). Chen et al. (1988) reported that rhizobia belong to several families of order rhizobiales of alpha Proteobacteria. Various historical developmental steps of rhizobial taxonomy are earlier reviewed by Jordan (1984), Graham et al. (1991), Young (1992), Elkan (1992) and Martinez-Romero and Caballero-Mellado (1996).

Molecular techniques such as sequence comparison of 16S rRNA genes have been used extensively for rhizobial characterization. By applying these technique many new nitrogen-fixing symbionts have been discovered in diverse genera such as *Methylobacterium, Burkholderia, Ralstonia (Cupriavidus), Ochrobactrum, Devosia* and *Phyllobacterium* (**Table 3**) (Chen et al. 2001; Van Berkum and Eardly 2002; Ngom et al. 2004; Valverde et al. 2005; Chen et al. 2006). Zakhia and de Lajudie (2001) classified the rhizobia with genera with 28 species. After that Sawada et al. (2003) reported 12 genera of rhizobia with 44 species from diverse legumes. In the second edition of the Bergey's Manual of Systematic Bacteriology (2005) rhizobia were categorised in different phylogenetic groups on the basis of 16S rRNA gene sequence analysis. In 2006, Willems reported 53 species of rhizobia. Few years later Berrada and Fikri-Benbrahim (2014) reported 98 species of legume nodulating bacteria belonging to 14 genera. Currently bacterial classification is based on modern polyphasic approach based taxonomy which also has very important role in classification of rhizobia that led to the description of many new genera and species (**Table 4**). This taxonomy is based on integration of all available phenotypic and genotypic data derived from 16S rRNA sequence analysis. This technique is very beneficial for the taxonomic assessment and diversity analysis of major bacterial groups. The exploration and increase in number of legumes from different geographical regions of world is also very determinative factor for description of non-reported member of rhizobia (Berrada and Fikri-Benbrahim 2014).

2.7 Recent taxonomy of rhizobia: Rhizobial diversity

Rhizobia are a heterogenous group of bacteria and classified by various taxonomists on the basis of many taxonomical parameters. Symbiotic bacteria associated with root nodules of legumes are currently divided into three main distinct phylogenetic subclasses: α, β and γ-Proteobacteria with 238 species that are grouped into 18 genera and two clades (**Fig. 7**) (Shamseldin et al. 2017).

Table 3: Increasing number of rhizobial genera

Year	Number of Rhizobial genera	References
1923	2	Bergey et al. (1923)
1984	3	Jordon (1984)
2001	6	Zhakhia and De Lazude (2001)
2003	12	Swada et al. (2003)
2014	14	Berrada and Fikri-Benbrahim (2014)
2017	18	Shamseldin et al. (2017)

The largest class, alpha-proteobacteria, is composed of six families, including *Rhizobiaceae, Phylobactericiae, Bradyrhizobiaceae, Hyphomicrobiaceae, Methylobacteriiaceae* and *Brucellaceae*. The second class beta-proteobacteria is comprised of two families of *Burkholderiaceae* and *Oxalobacteraceae* (Shamseldin et al. 2017). Root nodulating *Pseudomonas* is also reported which is the member of the γ-Proteobacteria and can nodulate *Robinia pseudoacacia* (Shiraishi et al. 2010). In another study Latif et al.

(2013) reported that a legume from tribe Trifoliate is nodulated by a sporulating Gram-positive Firmicutes *Paenibacillus sepulcri*.

Rhizobial taxonomy is defined by various methods and Ormeno-Orrillo et al. (2015) established a new trend of using whole genome sequence comparisons to define new taxa of rhizobia which is called as genomotaxonomy. Using this technique, they recently proposed new genus *Neorhizobium*, and suggested that *Rhizobium giardinii* be transferred to a novel genus. The recent revision of rhizobial taxonomy by Mousavi et al. (2015) led to the description of the novel genus *Pararhizobium*, comprised of 4 new species combinations and 9 other species combinations belonging to the genus *Allorhizobium*. *Pseudorhizobium pelagicum* is identified as a novel genus which had 95.6 to 97 % a sequence similarity to members of genera *Neorhizobium* and *Rhizobium*, but this new species lacked symbiosis-related genes (Kimes et al. 2015). Several study for species descriptions by whole-genome average nucleotide identity (ANI) and/or in silico DNA-DNA hybridization (DDH) have currently used (Delamuta et al. 2013). Application of these modern techniques have important role in increasing pace of modifications in rhizobia nomenclature that is increasing continuously with higher rate.

Molecular technique mainly 16S rRNA sequencing is highly used for rhizobia identification and diversity analysis but recently due to high level of sequence conservation, the application of the 16S rRNA gene as a phylogenetic marker is insufficient for species level resolution and phylogenetic analysis based on protein coding genes is more appropriate method for species level characterization (Glaeser et al. 2016). Other methods such as serology, RNA/DNA or DNA/DNA hybridization, analysis of plasmids etc. were used and led to new taxonomic studies of rhizobia (Rao et al. 2018). This opened the modern era, which has seen an increasing step of nomenclature change. Hence, it is clearly apparent that the current taxonomy of root nodulating rhizobia is rapidly progressing due to latest advances in molecular biology based omics technology e.g. genomics, proteomics, transcriptomics and metabolomics (Rao et al. 2018). Genotypic and phenotypic diversity of rhizobia is measured by a range of methods designed to collect data (Arora et al. 2018). These methods includes: classical phenotypic analysis and numerical taxonomy, whole cell protein analysis (multilocus enzyme electrophoresis- MLEE), analysis of cellular fatty acids (FAME), composition of DNA-base, pulsed field gel electrophoresis (PFGE), DNA-DNA reassociation, typing of IS (insertion sequence); RFLP (restriction fragment length polymorphism) of conserved genes (rRNA genes and intergenic spacer regions profiling), repC profiling, and whole genome fingerprinting using AFLP (amplified fragment length polymorphism), rep-PCR, random amplified polymorphic DNA (RAPD), apPCR (arbitrarily primed PCR) and DAF (DNA amplification fingerprinting) (Wang et al. 2006).

Because of their ecological and economic importance, the diversity and taxonomy of rhizobia have been extensively studied over the last 30 years (Evans 2015; Arora et al. 2018). From one genus including six species in 1974, the classification now includes at least 18 genera comprising more than 238 species and this number continues to increase (**Table 5**). The diversity of rhizobia from legumes is increasing annually and recent taxonomy and diversity of rhizobia is reviewed by various researchers such as Sahgal and Johri 2003, Rivas et al. 2009; Berrada and Fikri-Benbrahim 2014; Peix et al. 2015; Rao et al. 2018). The description of rhizobia species above comprises only about 23 % of legumes, and it has been estimated that there are roughly 19,000 legume species (Berrada and Fikri-Benbrahim 2014; Rao et al. 2018). The number of rhizobial species and their legume symbionts will increase in the future due to their wide geographical distribution in different ecosystems of the world major part of world including many geographical sites remain untouched and unexplored for legumes and rhizobia analysis.

Table 4: Increasing number of species in the genera of rhizobia (Willems et al. 2006; Mousavi 2016; Rao et al. 2018)

S. No.	**Major Rhizobial Genera**	**Number of Species**							
		1980	**1981-1985**	**1986-1990**	**1991-1996**	**1996-2000**	**2001-2006**	**2007-2015**	**2016-2018**
1.	*Agrobacterium*	4	4	5	5	5	5	5	98
2.	*Rhizobium*	6	7	7	10	10	16	72	
3.	*Bradyrhizobium*		1	1	3	3	7	27	37
4.	*Ensifer (Sinorhizobium)*			2	5	8	11	15	21
5.	*Azorhizobium*			1	1	1	2	3	3
6.	*Mesorhizobium*					7	11	30	40
7.	*Allorhizobium*					1	1	1	1
		10	**11**	**16**	**24**	**35**	**53**	**153**	**200**

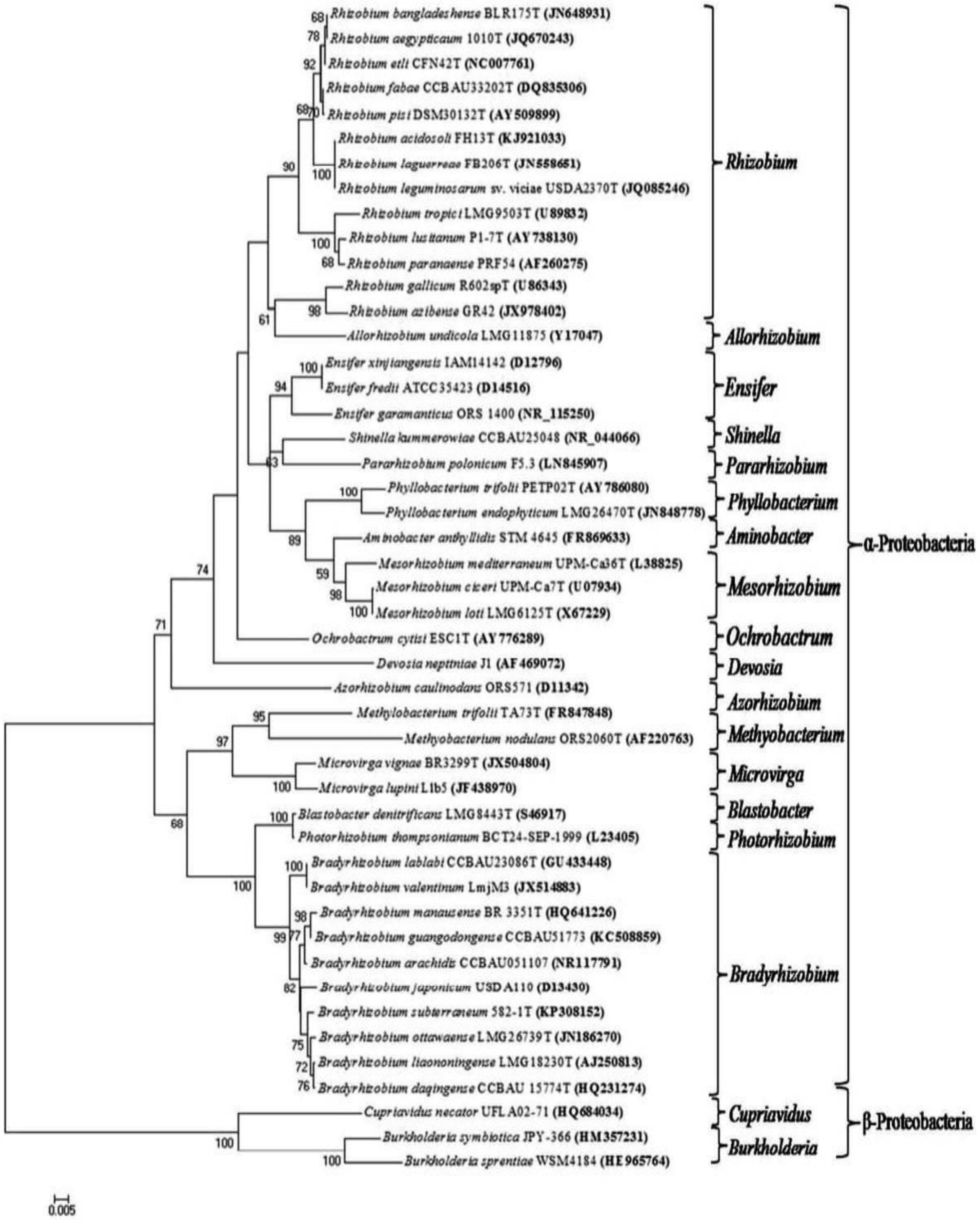


Fig. 7: Phylogenetic analysis of 16S rRNA sequence of representative species of 18 genera of nodule symbionts (Shamseldin et al. 2017)

Table 5: Recent taxonomy of rhizobia (Shamseldin et al. 2017; Rao et al. 2018)

Rhizobial strains	Number of species	Host Plants
Class α-Proteobacteria		
I Order *Rhizobiales*		
I Family *Rhizobiaceae*		
Rhizobium	**98**	Different plant hosts, Peas, Clover, Lupin, Soybean, *Lotus, Phaseolus, Astragalus,* Chickpea, *Sesbania, Medicago, Mimosa, Indigofera, Hedysarum, Medicago, Populus, Vicia, Lespedeza, Oryza, Albizzia, Kummerowia, Dalbergia, Caragana, Trigonella, Sphaerophysa, Oxytropis, Mung bean, Vigna, Rosa, Leucaena, Dalea, Clitoria, Siratro, Cowpea, Lemna, Calliandra, Pongamia, Arachis, Pueraria*
***Ensifer* (formerly *Sinorhizobium*)**	**21**	*Glycine, Sesbania, Acacia, Medicago, Prosopis, Kummerowia, Leucaena,* *Abrus, Lotus, Argyrolobium, Psoralea*
Genus *Allorhizobium*	**1**	*Neptunia*
Genus *Shinella*	**1**	*Kummerowia*
Genus *Pararhizobium*	**5**	Tumor of fruits (non-symbiotic)
II Family *Phyllobacteriaceae*		
Mesorhizobium	**40**	Different hosts, Chickpea, *Lotus, Astragalus, Leucaena, Sesbania,* *Amorpha, Prosopis, Albizzia, Biserrula, Caragana, Anthyliis, Robinia, Alhagi, Anagyris, Acacia, Sophora*
Phyllobacterium	**8**	*Lathyrus, Argyrolobium, Astragalus, Brassica, Phaseolus, Lotus, Sophora*
Aminobacter	**1**	*Anthyllis*
III Family *Bradyrhizobiaceae*		
Bradyrhizobium	**37**	*Glycine, Vigna, Lespedeza, Beta, Entada, Pachyrhizus, Lablab, Arachis,* *Cytisus, Retama, Aeshynomene, Acacia, Inga, Lupin, Phaesolus, Cowpea,* *Centrolobium, Erythrophleum, Neonotonia, Desmodium, Lupinus*
Blastobacter	**2**	*Aeschynomene*
Photorhizobium	**1**	*Aeschynomene*
IV Family *Hyphomicrobiaceae*		
Devosia	**1**	*Neptunia*
Azorhizobium	**3**	*Sesbania*
V Family *Methylobacteriaceae*		
Methylobacterium	**3**	*Crotalaria, Trifolium phyllosphere*
Microvirga	**4**	*Lupinus, Listia, Cowpea*
VI Family *Brucellaceae*		
Ochrobacterium	**2**	*Lupinus, Cytisi*
Burkholderiales		
Family *Burkholderiaceae*		
Burkholderia	**17**	*Dalbergia, Machaerium, Mimosa, Lebeckia, Aspalathus, Papilionoid*
***Cupriavidus* (former *Ralstonia*)**	**2**	*Mimosa, Phaselous, Leucaena*

2.8 Rhizobia as PGPR: Rhizobial inoculants

Rhizobia have the important ability to fix atmospheric nitrogen in symbiotic association with legumes (Singha et al. 2017). Besides nitrogen fixation rhizobia also have many direct and indirect plant growth promoting characters such as production of phytohormones (Sridevi et al. 2009), lipochitooligosaccharides (LCOs) (Dyachok et al. 2000), lumichrome (Gouws et al. 2012), siderophores (Arora and Verma 2017), hydrogen cyanide (HCN) (Manasa et al. 2017), ACC deaminase (Kumar et al. 2016). exopolysaccharides (EPS) (Bashan et al. 2014; Kumari et al. 2018), phosphate solubilizing enzymes (Bhargava et al. 2016) and biocontrol activity against phytopathogens (Aeron et al. 2017) which could improve plant growth and yield. Due to above reported properties rhizobia are used as bioinoculants in agriculture for high crop productivity (**Table 6**) (Laranjo et al. 2014; Arora et al. 2017). Application of these PGPR based inoculants on crop can be very helpful effort for food security as well as agricultural sustainability.

Rhizobia are used as crop inoculant since very ancient times. Before the discovery of rhizobia, the inoculation of seed or soil was done in the crop by “soil transfer method”, in which soil from legume grown field to field or field to seed were applied before planting (Fred et al. 1932). It is reported that if any soil have less amount of specific and effective rhizobia or lack of it, then introduction of rhizobial inoculants is required in that soil for appropriate nodulation along with nitrogen fixation. Inoculation of legume is an efficient way of introducing effective rhizobia to soil and subsequently the rhizosphere of legumes. The application of rhizobial inoculant is one of the cheapest and eco-friendly approaches for improving production of leguminous plants (Thakare and Rasal 2000). It has been estimated that 2,000 tons of rhizobial inoculants of worth US$ 50 million are produced worldwide every year (Ben Rebah et al. 2007) and this quantity is sufficient to inoculate 20 million hectares of legumes (Herridge et al. 2002). Rhizobial inoculants have long been incorporated into field practices worldwide, with satisfactory results (Somasegaran and Hoben 1985; Thuita et al. 2012). Application of rhizobial inoculants enhances nodulation, crop yield and biomass of the plants (Afzal and Bano 2008). Rhizobial inoculation also enhances the bioactive compounds of legumes with biomass which has important role in human health (Prabha et al. 2013; Silva et al. 2017). It is estimated that efficient strain of rhizobial inoculant can enhance the crop yield up to 10-35% since N is fixed at 40-200 kg/ha which reach up to 80-90% of N requirement of the crop (Mazid and Khan 2014).

Rhizobial inoculants are highly being used as unique plant growth promoting rhizobacteria for leguminous plants as well as non-leguminous plants and have potent role in growth enhancement of plants (Deshwal et al. 2013; Arora et al. 2017; Ramesh and Reddy 2018). The demand of rhizobial bioinoculants is high and these are produced commercially at global level (Arora et al. 2017). Rhizobial inoculants are very inexpensive source of biofertilizers as eco-friendly source (Ghimire 2002). Exploration and identification of several pant growth promoting rhizobial species can be efficiently used as growth enhancer of edible common edible legumes to fulfil future nutritional needs of humans. However rhizobial inoculants are being used globally since long time, but still there is scope for their improvement so as to enhance the efficacy, productivity and reliability amongst the farmers (Arora et al. 2017). Mining of rhizobial diversity from unexplored legumes is considered as a valuable biological resource and has important role to find the more efficient bioinoculants having broad range of plant growth promoting attributes and efficient combinations of rhizobia-legume symbiosis.

Table 6: Rhizobial inoculants (Berrada and Fikri-Benbrahim 2014; Arora et al. 2017)

Genus	Species	Inoculant Crop	References
Rhizobium	*leguminosarum*	*Lactuca sativa and Daucus carota, Pea*	Flores-Félix et al. (2013); Clayton et al. (2004b)
	galegae	*Galega orientalis*	Vassileva and Ignatov (2002)
	tropici	*Zea mays*	Marks et al. (2015)
	endophyticum	*P. vulgaris*	López-López et al. (2010)
	phaseoli	*Vigna radiata*	Zahir et al. (2010)
	fabae	*V. faba*	Tian et al. (2008)
	etli	*P. vulgaris*	Soares et al. (2006)
	undicola	*Neptunia natans*	de Lajudie et al. (1998)
	gallicum	*P. vulgaris*	Sassi-Aydi et al. (2012)
	giardinii	*P. vulgaris*	Amarger et al. (1997)
	hainanensis	NA	NA
	huautlense	*Sesbania herbacea*	Wang and Martı´nez-Romero (2000)
	mongolense	*Medicago ruthenica*	Van Berkum et al. (1998)
	yanglingense	NA	NA
	larrymoorei	NA	NA
	indigoferae	NA	NA
	sullae	*Hedysarum coronarium*	Fitouri et al. (2012)
	loessense	NA	NA
	cellulosilyticum	*P. vulgaris*	Diez-Mendez et al. (2015)
	miluonense	*Lespedeza chinensis*	Gu et al. (2007)
	multihospitium	NA	NA
	oryzae	*Glycine max*	Waswa (2013)
	pisi	NA	NA
	mesosinicum	NA	NA
	alamii	*Helianthus annuus*	Alami et al. (2000)
	alkalisoli	NA	NA
	tibeticum	*Trigonella foenumgraecum*	Abd-Alla et al. (2014c)
	tubonense	NA	NA
	halophytocola	NA	NA

	radiobacter	*Graminaceous crops*	Humphry et al. (2007)
	rhizogenes	NA	NA
	rubi	NA	NA
	vitis	NA	NA
	nepotum	NA	NA
Ensifer	*meliloti*	*Medicago truncatula, Mucuna pruriens*	Olah et al. (2005); Aeron et al. (2012)
	fredii	*G. max*	Albareda et al. (2008)
	sahelense	NA	NA
	terangae	NA	NA
	medicae	NA	NA
	arboris	NA	NA
	kostiense	NA	NA
	xingianens	NA	NA
	adhaerens	NA	NA
	kummerowiae	NA	NA
	americanum	*P. vulgaris*	Mnasri et al. (2012)
	mexicanus	*P. vulgaris*	Lloret et al. (2007)
	numidicus	NA	NA
Shinella	*kummerowiae*	NA	NA
Mesorhizobium	*loti*	*Lotus corniculatus*	Karaś et al. (2015)
	huakuii	NA	NA
	cicero	*Cicer arietinum*	Rokhzadi et al. (2008)
	tianshanense	NA	NA
	mediterraneum	*Hordeum vulgare, Cicer arietinum*	Peix et al. (2001); Dudeja et al. (2011)
	plurifarium	NA	NA
	amorphae	NA	NA
	chacoense	NA	NA
	septentrionale	NA	NA
	temperatum	*G. max*	Waswa (2013)
	thiogangeticum	NA	NA
	albiziae	*Albizia kalkora, G. max*	Wang et al. (2007), Waswa (2013)
	caraganae	NA	NA

	gobiense	NA	NA
	tarimense	NA	NA
	australicum	NA	NA
	opportunistum	NA	NA
	metallidurans	NA	NA
	alhagi	NA	NA
	camelthorni	NA	NA
	abyssinicae	NA	NA
	muleiense	NA	NA
	hawassense	NA	NA
	qingshengii	NA	NA
	robiniae	NA	NA
	shonense	NA	NA
	shangrilense	NA	NA
	silamurunense	NA	NA
	tamadayense	NA	NA
Phyllobacterium	*trifolii*	*Fragaria ananassa*	Flores-Felix et al. (2015)
Methylobacterm	*nodulans*	*Crotalaria perrottetii*	Jourand et al. (2004)
Microvirga	*lupine*	NA	NA
	lotononidis	*Leobordea sp.*	Ardley et al. (2013)
	zambiensis	NA	NA
Ochrobactrum sp.	*cytisi*	*Cucumis sativus*	Xu et al. (2015)
	lupine	*Lupinus albus*	Trujillo et al. (2005)
Azorhizobium	*caulinodans*	*Leucaena leucocephala*	Waelkens et al. (1995)
	dobereinereae	NA	NA
	oxalatiphilum	NA	NA
Devosia	*neptuniae*	*Neptunia natans*	Rivas et al. (2003)
Bradyrhizobium	*japonicum*	*G. max*	Zerpa et al. (2013)
	elkanii	*Vigna unguiculata*	Soares et al. (2006)
	iaoningensese	NA	NA
	yuanmingense	*G. max*	Soe and Yamakawa (2013)
	betae	NA	NA
	canariense	NA	NA

	iriomotense	NA	NA
	jicamae	NA	NA
	lablabi	NA	NA
	huanghuaihaiense	NA	NA
	cytisi	NA	NA
	daqingense	NA	NA
	denitrificans	NA	NA
	oligotrophicum	NA	NA
	pachyrhizi	NA	NA
Burkholderia	*caribensis*	*Amaranthus cruentus and A. hypochondriacus*	Parra-Cota et al. (2014)
	cepacia	*P. vulgaris*	Peix et al. (2001)
	tuberum	*Macroptilium atropurpureum*	Annette et al. (2013)
	phymatum	*P. vulgaris*	Talbi et al. (2013)
	nodosa	NA	NA
	sabiae	NA	NA
	mimosarum	NA	NA
	rhizoxinica	NA	NA
	diazotrophica	NA	NA
	endofungorum	NA	NA
	heleia	NA	NA
	symbiotica	*Mimosa cordistipula*	Sheu et al. (2012)
	ambifaria	*Zea mays, A. cruentus and A. hypochondriacus*	Ciccillo et al. (2002); Parra-Cota et al. (2014)
	vietnamiensis	*Oryza sativa*	Choudhury and Kennedy (2004)
Cupriavidus	*taiwanensis*	*Rhynchosia ferulifolia*	Garu et al. (2009)
Pseudomonas	NA	NA	Zhao et al. (2013)

(NA= Not Available)

2.9 Non-rhizobia

Root nodules of leguminous plants show symbiotic association with rhizobia which is a group of root nodulating bacteria. Root nodules of leguminous plants are very rich in nutrients which also attract various types of non-rhizobial and nonsymbiotic bacteria to colonize plants opportunistically (Wang et al. 2018). Root nodules are also providing a protective environment for the bacteria to live in them (Sprent 2009). In many studies various types of fast growing non-rhizobial bacteria have been isolated from root nodules with or without rhizobia (Rajendran et al. 2012; Aeron et al. 2015). Rhizobia live inside the nodule with various non-rhizobial bacteria (Martinez-Hidalgo and Hirsch 2017). These non-rhizobial isolates are also considered as contaminants of root nodules and known by various names such as nodule associated bacteria (NAB) (Rajendran et al. 2012), nodule endophytes (NE) (Velazquez et al. 2013), non-rhizobia endophytes (NRE) (De Meyer et al. 2015). These nodule endophytes are being isolated from various legumes such as soybean, mung bean, groundnut, cowpea, kudzu, aprajita, acacia, fenugreek, alfalfa (Selvakumar et al. 2008; Rajendran et al. 2012; Dhole 2017; Aeron et al. 2015; Boukhatem et al. 2016; Cinnaswamy et al. 2018). These non-rhizobial endophytes belong to various bacterial genera such as *Bacillus* (Xu et al. 2014), *Pantoea* (De Meyer et al. 2015), *Paenibacillus* (Latif et al. 2013), *Pseudomonas* (Bouchiba et al. 2017), *Enterobacter* (Benhizia et al. 2004), *Serratia* (Selvakumar et al. 2008), *Staphylococcus* (Xxu et al. 2014), *Microbacterium* (Boukhatem et al. 2016), *Exiguobacterium* (Rajendran et al. 2012), *Acinetobacter* (Zhao et al. 2017). These bacteria live in nodules more than just as opportunistic colonizers (Selvakumar et al. 2008). In various studies non-rhizobial isolates are more predominantly reported than true symbiotic rhizobia from root nodules of legumes (Palaniappan et al. 2010; Deng et al. 2011; Aeron et al. 2015). These bacterial genera are from most common soil inhabiting rhizospheric bacteria. The entry of non-rhizobial bacteria into nodules is still not clearly identified but they appear to be opportunistic in nature. However, their exact work in nodules is not well known but some studies reported that they help the rhizobia in nitrogen fixation. These endophytes are also reported as efficient plant growth promoters (Aserse et al. 2013; Dixit et al. 2014; Sansanwal et al. 2018) and can be exploited for high crop production in agriculture.

Legumes are associated with large number of bacteria which are clearly known but we can state that there is chance to know the more diverse bacteria than already reported isolates which is either rhizobia or non-rhizobia with more potent plant growth promoting activities. Exploration of legumes and their bacterial associates can be used for the solutions of various environmental problems in sustainable way hence there is urgent need of mining of rhizobial as well as non-rhizobial diversity from unexplored mainly wild medicinal legumes growing in waste and uncultivated sites of not only India but all over the world.

MATERIALS AND METHODS

3.1 Collection of sample (wild medicinal legumes):

In this study five wild medicinal legumes *Abrus precatorius, Crotalaria juncea, Clitoria ternatea*, *Leucaena leucocephala* and *Sesbania bispinosa* were selected for isolating the symbiotic rhizobia associated with their root nodules. Plants (wild medicinal legumes) were collected randomly from different regions of Lucknow (26.8467° N, 80.9462° E) and adjoining areas such as Barabanki (26.9955° N, 81.2519° E), Unnao (26.5393° N, 80.4878° E) and Kanpur (26.4499° N, 80.3319° E) from the state of Uttar Pradesh, India. Sampling sites were mainly waste and uncultivated lands. The intact root system bearing nodules of plant was uprooted carefully and after cutting off the shoot portion plants were aseptically placed in plastic/polypropylene bags, and immediately brought to laboratory for isolation of bacteria according to Somasegaran and Hoben (1984).

3.2 Analysis of internal structure of root nodules:

Internal structure of root nodule was observed by microscopy. Fresh root nodules were taken and cut as very thin slice and observed under light microscope. It was also observed under electron microscopy at University Scientific Instrumentation Centre (USIC), BBA University, Lucknow.

3.3 Isolation of root nodulating bacteria:

Root samples were properly washed with tap water to remove adhering soil. After washing healthy nodules of each plant root was detached carefully and used for bacterial isolation. Firstly, root nodules were washed with tap water, then surface sterilized with 70% alcohol for 3 min and after that rinsed with 0.1 % mercuric chloride for another 3 min. Nodules were washed several times (5-6) with sterile distilled water to remove the effect of sterilizing agents (Somasegaran and Hoben 1994). The nodules of each plant were crushed with sterile rod in a drop of sterile distilled water in separate petri dishes. A loop-full of the each suspension was then streaked on congo red yeast extract mannitol agar (CRYEMA) media plates (Hi Media, Mumbai) and incubated at 28 ºC for 3-5 days (Vincent 1970). Bacterial isolates were streaked several times on yeast extract mannitol agar (YEMA) to obtain pure culture. Pure bacterial isolates were maintained on YEMA slants and stored at 4 °C and also in 25% glycerol stock solution at -80 ºC (Labocon, LUF-86-100). All rhizobial isolates were re-plated on YEMA media and regularly checked for contamination every three months.

3.4 Phenotypic characterization:

The bacterial isolates were characterized according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and Bergey's Manual of Systematic Bacteriology (Garrity et al. 2005) by various standard methods.

3.4.1 Morphological characterization:

i. Colony morphology:

Colony morphology of the isolates was determined by observing their growth on CRYEMA media plates. Each isolate was grown on solid CRYEMA/YEMA media plates and observed for the growth pattern, colour, shape, size, elevations and texture of their colonies (Vincent 1970; Khandelwal 2002).

ii. Cell morphology:

Cell morphology of the bacterial isolates was determined by Gram staining and electron microscopy. Gram staining of isolates was done according to Gram (1884). Clean slide was taken and smear of fresh culture of each bacterial isolates was prepared. Bacterial smear was flooded with crystal violet stain for 60 seconds then rinsed with distilled water. After this few drop of Gram’s iodine was put on the smear for 60 seconds, followed by washing with 95% alcohol drop by drop until the purple dye no longer flows from the smear and again washed gently with water. In last the smear was flooded with a counter stain safranin for 30 seconds and again washed with distilled water. After air drying the slide was observed under microscope for determining the shape and Gram's nature of the isolates.

Cell morphology (size and shape) of the isolates were also determined by observing them under electron microscope (JEOL, JSM-6490LV) at USIC, BBA University, Lucknow.

3.4.2 Physiological and metabolic characterization:

i. Bromothymol blue (BTB) test:

This test was done to determine the isolates either fast growers or slow growers which was done on YEMA plates supplemented with 0.25 mg/L BTB as indicator dye (Norris 1965). Plates were spot inoculated with bacterial culture and incubated for 7 days at 30 °C and observed for colour change. The change of colour from green to blue indicated alkali and green to yellow indicated acid production. Colony appearance was also recorded either as dry (smooth and firm surface) or wet (watery or slimy).

ii. Generation time:

Generation time of isolates was determined by inoculating a loopful log phase culture separately in 50 ml sterilized YEM broth and incubated at 28 °C for 24 h at 150 rpm. Bacterial growth of each isolate was assessed by measuring the optical density at 600 nm after every 6 h in UV-VIS spectrophotometer (Thermo Scientific, Evolution 201) (Dubey and Maheshwari 2012). The generation time was calculated using the following formula:

$$\text{Generation time} = (T2–T1)/\ 3.3\ (\log_{10} OD2 - \log_{10} OD1)$$

Where (T2-T1)= difference of two time intervals at any two point in log phase in growth curve; ($\log_{10}$ OD2- $\log_{10}$ OD1)= difference between the $\log_{10}$ value of OD2 at time T2 and OD1 at time T1.

iii. Motility:

Motility of the isolates was determined by observing their growth pattern in semisolid motility test agar media (Tittsler and Sandholzer 1936). Motility medium was prepared and inoculated with needle by stabbing in test tubes. Inoculated tubes were incubated at 28 ºC for 24 hrs and then observed for the bacterial growth to determine their motility.

iv. Utilization of different carbon sources:

All the isolates were tested for their ability to utilize different carbon sources such as mannitol, glucose, dextrose, lactose, galactose, sucrose, maltose, starch, glycerol, fructose, citrate, malate and trehalose. In this test mannitol was replaced with other carbon sources from YEMA medium (Zablotowicz and Focht 1981). Each carbon source was sterilized by filtration through Millipore membranes (pore size 0.22 μm) and added in medium to final concentration of 10% (w/v). Media was poured on plate and streaked with each isolate and observed their growth after incubation at 28 °C for 24 to 48 hrs.

v. Utilization of different nitrogen sources:

Ability of isolates to utilize various nitrogen sources was done on YEMA plate by replacing yeast extract from media with various nitrogen sources such as yeast extract, potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), ammonium sulphate (NH_4SO_4), glutamine, tryptophan, glycine, cysteine, lysine and methionine (Holt et al. 1994). Plates of each media were prepared and inoculated with loop full of culture and incubated for 24 to 48 hrs at 28 ºC temperature. After incubation growths of isolates were observed.

vi. Growth at different salt concentrations:

Salt tolerance activity of isolates was determined at various salt concentrations ranged from 1 to 10% (Chen et al. 1988). This was done on YEMA media plates amended with various salt concentrations. Plates were streaked with log phase bacterial cultures (10^8 cfu/ml) and incubated at 28±1 °C for 3 days. Growth of isolates was recorded after incubation.

vii. Growth at different pH:

The pH tolerance activity of isolates was determined in YEMA media adjusted to pH 5.0–12.0 (Zablotowicz and Focht 1981). The pH of the medium was adjusted by the addition of

HCl or NaOH before sterilization. Plates were inoculated with log-phase culture (10^3 cfu/ml) and incubated at 28±1 °C for 3-5 days.

viii. Growth at different temperature range:

The effect of temperature on growth of isolates was observed on YEMA plates. Plates were streaked with log-phase cultures (10^8 cfu/ml) and kept for 3 days at various temperatures ranging from 4 to 44 °C. After incubation growth of isolates at each temperature was observed (Pandey et al. 2006).

ix. Intrinsic antibiotic resistance (IAR):

The IAR activity of isolates is also useful for determination of their diversity (Arora et al. 2018). This test was done according to Kirby-Bauer method or disc diffusion method (Bauer et al. 1996) on YEMA media plates. Firstly suspension of each bacterial culture was prepared and spread on YEMA plates for lawn formation and discs of various antibiotics (Himedia, Mumbai) were placed over it. Four discs were placed in each plate and incubated for 48 hrs at 28 ºC and observed the clear zone formation around the discs. Size of clear zone formation was measured and used to calculate antibiotic sensitivity index of the isolates. Various antibiotics such as gentamicin (GEN 10mcg), streptomycin (S 10 mcg), nalidixic acid (NA 30 mcg), ampicillin (AMP 10mcg), neomycin (N 30mcg), kanamycin (K 30mcg), erythromycin (E 15mcg), penicillin (P 10mcg), tetracycline (TE 30mcg) and chloramphenicol (C 30 mcg) were used.

3.4.3 Biochemical characterization:

i. Catalase test

In this test a loopfull of culture was kept on glass slide and 2-3 drops 3% hydrogen peroxide (H_2O_2) was poured over it and slides were observed for the liberation of effervescence of oxygen around the bacterial colonies as bubble formation (Graham and Parker 1964).

ii. Oxidase test

This test was performed by using oxidase discs saturated with Kovacs reagent (1% aqueous tetra methyl-para-phenylene-diamine solution) (Kovacs 1956). This disc was placed on YEMA plates and on to them fresh bacterial culture cultures were spotted. Appearance of dark purple colour was observed within 10 second for positive oxidase test.

iii. Lactose test

For this test lactose agar medium plates were prepared, spot inoculated with bacteria and incubated for 24-48 hrs at 28 ºC. After incubation, Benedict’s reagent was added over the plates and observed the formation of yellow coloured zone for positive lactose test (Bernearts and De Ley 1963).

iv. Growth on Glucose peptone agar (GPA):

GPA media was prepared and bacterial cultures were streaked on it and incubate for 48 hrs at 28 ºC. After incubation growth of isolates was observed (Singh et al. 2008).

v. Growth on Hoffers alkaline media (HAM):

Hoffer’s alkaline agar media was prepared and inoculated with fresh bacterial culture (Hoffer 1935). After inoculation plates were incubated for 2-3 days at 28 ºC to observe the bacterial growth.

vi. Growth in presence of 8% potassium nitrate (KNO_3):

In this test bacterial culture were grown in YEM broth media supplemented with 8% (w/v) potassium nitrate (KNO_3) and observe their growth after 48 hrs at 28 ºC (El Idrissi et al. 1996).

vii. Nitrate reductase activity

This test was performed on tryptone yeast extract (TYE) medium containing 0.1 % KNO_3 (Campbell 1999). Bacterial isolates were inoculated and incubated for 4 days at 28 ºC. After incubation few drops of sulphanilic acid (8 g/l) in 5M acetic acid) and α-naphthylamine (5 g/l in 5M acetic acid) were added in each tubes including control. Colour change was observed for result analysis.

viii. Urease test

YEMA medium was prepared in which 2% (w/v) urea and 0.012% (w/v) phenol red was added (Lindstrom and Lehtomaki 1988). Slants of the urea containing medium was prepared for each bacterial culture including control which was inoculated with bacterial culture and incubated at 28 ºC for 48 hrs. Colour change of media from light yellow to red was observed for urease production.

ix. Gelatinase test

Gelatinase media was prepared and poured in separate test tubes for each isolates including one control. All the tubes were stab inoculated with inoculating needle and incubated for 3-5 days at 28 ºC. After incubation the test tubes were placed in refrigerator at 4 ºC for 15 minutes and observed. Partial or total liquid state of gelatin confirmed the positive test (Chapman 1952).

x. Citrate utilization test

In this test slant of Simmon's citrate agar media was prepared in test tube and inoculated with bacterial culture by streaking on it (Simmon 1926). All tubes including one uninoculated control was incubated at 28 ºC for 24 hrs and observed for the colour change from green to blue.

xi. Amylase test:

Starch agar media was prepared and spot inoculated with bacterial culture and incubated at 28 ºC for 48 hrs (Lennette et al. 1985). After incubation plates were flooded with Gram's iodine solution for 4-5 minute. After removing the extra iodine solution, plates were observed for the clear zone around colonies.

xii. Lipase test

Lipase test was done on Tween 80 agar media (Samad et al. 1989). Media plates were prepared, inoculated and incubated for 2-3 days at 28 ºC. After incubation opaque zone formation was observed around the bacterial colonies on transparent media.

xiii. Protease test:

For this test skim milk agar media was prepared, inoculated and incubated for 48 hrs at 28 ºC. After incubation clear zone formation was observed around the bacterial colonies (Kasana et al. 2011).

xiv. Cellulase test:

Czapek's mineral salt agar media was prepared, inoculated and incubated for 2-3 days 28 ºC. After the growth of the colonies, plates were flooded with dye (congo red) for 15 minutes for staining and 1% NaCl for destaining for 15 minutes. After destaining hollow zone formation around the bacterial colonies was observed (Florencio et al. 2012).

xv. Ammonia production:

Peptone broth was prepared and dispensed 5 ml broth in various tubes for each bacterial culture. Tubes were inoculated with bacteria and incubated for 48 hours at 28 ºC. After incubation 0.5 ml Nessler's reagent was added in each test tube along with the control to observe colour change with slight precipitation (Cappuccino and Sherman 1992).

xvi. Polyhydroxy butyrate (PHB) production

Each bacterial isolates were streaked on YEMA plates and incubated at 28 ºC for 48 hrs. When bacterial growth was appeared ethanolic solution of 0.3% (w/v in 70% ethanol) Sudan Black B dye was spread over the colonies for 30 minutes and destained by washing with ethanol (96%) to remove excess stain from the colonies. The colonies that retained their black colour were identified as PHB producing strains (Mohamed et al. 2012).

xvii. Indole test:

Tryptone broth was prepared and inoculated with loopfull of culture and incubated for 24 hrs at 28 ºC. Then after incubation 1 ml of Kovac's reagent was added to each tube including control and the tubes were observed for the formation of "Red colour ring'' on the top of the broth (Cheesbrough 1985).

xviii. Methyl Red-Voges Proskauer (MR-VP) test:

The MRVP broth was prepared and transferred 10-10 ml in separate tubes. Each tube was inoculated with one loopfull bacterial culture and incubated for 24 hrs at 28 ºC. After incubation media of each test tubes was equally divided into 2 test tubes one for MR test and another for VP test. For MR test 5 drops of methyl red was added to one set of tube and for VP test 12-15 drops of VP- I (5% α- naphthol) and VP- II (40% potassium hydroxide) was added to another set of test tubes. The sample was kept for 10-15 minutes to complete the reaction then the result was observed on the basis of colour change (Faddin and Jean 2000).

3.5 Plant growth promoting characterization:

After morphological, biochemical and physiological characterization of bacterial isolates, their plant growth promoting potential was also determined by performing various tests.

3.5.1 Nitrogen fixation:

Nitrogen free Jensen's medium was used for this assay (Jensen 1942). This medium is recommended for detection and cultivation of nitrogen fixing bacteria. Solid agar media plate was prepared and inoculated with bacterial culture. After inoculation plates were incubated for 3-7 days at 30 ºC and observed for the growth of colonies.

3.5.2 Phosphate solubilisation:

This assay was done on plates containing Pikovskaya's agar media. Solid agar media plates were prepared and spot inoculated with fresh bacterial culture (Pikovskaya 1948). After inoculation plates were incubated at 28 °C for 2-4 days and observed for the halo zone formation around the bacterial colonies. Halo zone formation was used for the determination of phosphate solubilisation index (PSI) by applying the following formula (Edi-Premono et al. 1996).

$$\text{PSI} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

3.5.3 Zinc solubilisation:

This assay was done on Zn solubilising basal agar medium amended with 0.1% insoluble zinc sources (zinc oxide-ZnO, zinc carbonate- $ZnCO_3$ and zinc phosphate- $Zn_3(PO4)_2$). Media plate was prepared and spot inoculated with bacterial culture and incubated at 28±1 ºC for 3-5 days (Fasim et al. 2002). After incubation clear zone formation around the grown bacterial colonies were observed on plates. Clear zone was measured and used for the calculation of zinc solubilisation index (ZSI).

$$\text{ZSI} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

3.5.4 HCN production:

HCN production ability of isolates was determined according to Bakker and Schippers (1987). For this test HCN induction solid agar media plates were prepared by adding 4.4 g/l glycine in Luria Bertani (LB) agar and each bacterial culture was spread on separate plate. One uninoculated plate was used as control. Filter paper (Whatman No. 1) dipped in picric acid solution (2% sodium carbonate mixed with 0.5% picric acid solution) was placed in the lid of petri plates. All the plates were sealed with parafilm and incubated at 28 ºC for 4 days. After incubation colour change of filter paper from yellow to brown was observed against control plate.

3.5.5 Siderophore production:

The detection of siderophore production ability of isolates was done by universal CAS (chrome-azurol sulfonate) assay (Schywn and Neilands 1987). Firstly glassware used for this test was rinsed with 3M hydrochloric acid (HCl) to remove iron and subsequently washed in deionized water (Cabaj and Kosakowska 2007). Siderophore production was estimated both qualitatively and quantitatively. CAS reagent was prepared according to Schwyn and Neilands (1987). Briefly, 121 mg CAS dye was dissolved in 100 ml distilled water and 20 ml of 1 mM ferric chloride ($FeCl_36H_2O$) solution prepared in 10 mM HCl. This solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution (729 mg HDTMA in 400 ml distilled water) with continuous stirring. This is called as CAS-HDTMA solution which was sterilized before further use.

i. Qualitative assay:

This was performed according to modified method given by Hu and Xu (2011). CAS agar plates were prepared by mixing 100 ml CAS reagent in 900 ml sterilized LB agar medium. Four bacterial strains were spotted on each plate. An uninoculated plate was taken as control. After inoculation, plates were incubated at 28 ºC for 5-7 days and observed orange coloured zone formation around the bacterial colonies (Louden et al. 2011).

ii. Quantitative assay:

A) Traditional method:

Quantitative estimation of siderophore production was done by using supernatant of bacterial cultures grown in LB broth medium (Hu and Xu 2011). For each bacterial culture 1 ml broth was taken in 1.5 ml centrifuge tube (Thomas Scientific, US) and after sterilization inoculated with freshly grown bacterial culture (10^8 cfu/ml). Tubes were incubated at 28°C for 48 hrs and after incubation bacterial cultures were centrifuged (10,000 rpm for 10 min) (REMI, CM-12 Plus). Bacterial supernatant was used to estimate the siderophore production. Supernatant (0.5 ml) of each bacterial culture was mixed with equal amount (0.5 ml) of CAS reagent and after 20 min OD was taken at 630 nm (Thermo Scientific, Evolution 201). Siderophore produced by strains were measured in percent siderophore unit (psu) which was calculated according to the given formula (Payne 1993):

$$\text{Siderophore production (psu)} = \frac{(Ar-As)\ x100}{Ar}$$

Where Ar = absorbance of reference (CAS solution and un-inoculated broth), As = absorbance of sample (CAS solution and cell free supernatant of sample)

B) Modified microplate method:

The siderophore production was also checked by modified microplate method which was done by using 96 well- microtiter plate (Arora and Verma 2017). In this method 100 µl of each bacterial supernatant was added in the wells of microplate (Sigma, CLS3474) and 100 µl CAS reagent was also added in each well including uninoculated control. After incubation (20 minutes) OD of each sample was recorded using microplate reader (Spectra, Max M5e) at 630 nm. Siderophore production was estimated by the same formula as mentioned above.

3.5.6 Gibberellin production:

Gibberellin production ability of isolates was determined by the protocol of Holbrook et al (1961). Bacterial culture was inoculated in YEM broth medium and incubated at 28 ºC for seven days in shaking condition (150 rpm). After bacterial growth 7.5 ml of culture supernatant was taken and 1 ml of zinc acetate reagent was added. Zinc acetate reagent was prepared by mixing 21.9 g zinc acetate with 1 ml of glacial acetic acid and volume made to 100 ml with distilled water. After 2 minutes 1 ml of potassium ferrocyanide (10.6% in distilled water) was added in each tube and centrifuged (REMI, CM-12 Plus) at 2000 rpm for 15 minutes. After that 2.5 ml of supernatant was taken and 2.5 ml of 30 % HCl was added in it. This mixture was incubated at 27 °C for 75 minutes and absorbance was taken at 254 nm and concentration of gibberellins was calculated by using standard curve of pure gibberellic acid (Sigma-aldrich) (Holbrook et al 1961).

3.5.7 IAA production:

IAA production ability of isolates was checked by colorimetric analysis (Gordon and Weber 1951). In this method YEM broth media amended with 0.5 % tryptophan was prepared and inoculated with loopful of fresh grown bacterial culture and incubated for 4 days at 28 ºC. After incubation culture was centrifuged (10,000 rpm for 30 min) and supernatant was used for IAA estimation. After that 1 ml of supernatant was taken and 2 ml of Salkowaski reagent (1.0 ml of 0.5 M ferric chloride- $FeCl_3$, 50 ml of distilled water and 30 ml of concentrated sulfuric acid-H_2SO_4) was added in it and observed for the appearance of red/pink colour after 30 minute incubation (Salkowski 1885). OD of each sample including control was taken at 530 nm (Thermo Scientific, Evolution 201). Standard graph of pure IAA (Sigma-aldrich) was also prepared by plotting concentration of IAA (10-120 µg/ml) in µg/ml vs OD at 530 nm. It was prepared to calculate the IAA produced by bacterial isolates.

Quantitative estimation of IAA production was also done by modified microplate method (Sarwar and Kremer 1995; Arora and Verma 2017). In this method 100 µl supernatant of each bacterial culture grown in tryptophan supplemented media was dispensed in well of microplate (CLS3474 Sigma) and 100 µl Salkowski reagent was also added in each well and incubate the plate for 30 minute. After incubation optical density of each sample placed in wells of microplate was recorded in microplate reader (Spectra, Max M5e) at wavelength of 530 nm. All samples including control (without bacterial culture) were estimated in four replications in 96 well plate and calculated according to standard curve of pure IAA.

i. Optimization:

Various growth parameters were optimized for IAA production by using tryptophan supplemented YEM broth medium (Gordon and Weber 1951). Optimization was done at various cultural conditions such as pH (4, 5, 6, 7, 8), carbon sources (glucose, lactose, mannitol, mannose and sucrose), nitrogen sources (yeast extract, glycine, ammonium chloride, sodium nitrate and potassium nitrate), tryptophan concentrations (.05-0.4%), incubation period (24, 48, 72, 96, 120 hrs) and salt concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%). YEM broth was used for all the experiments incubated for 72 hrs at 28 ºC temperatures. After the completion of incubation period OD was taken at 530 nm and IAA production was estimated and compared.

ii. Extraction and purification:

For extraction process 100 ml of YEM broth media was prepared in 250 ml conical flask at optimized conditions, which was inoculated with loopful of bacterial culture and incubated for 4 days at 28 ºC (Nitsch 1955). After growth culture medium was centrifuged (10,000 rpm for 30 min) and supernatant was collected. After that pH of the supernatant was maintained at 2.5 by adding 1N HCl and mixed with ethyl acetate (1:2). After vigorous shaking it was allowed to stand for 10 min. Ethyl acetate was evaporated at 40 °C and IAA was extracted within solvent layer. This procedure was repeated 3 to 4 times. The extract was dissolved in methanol and kept at 20 °C for further characterization.

After extraction, IAA sample was purified by thin layer chromatography (TLC) (Datta and Basu 1998). In this process TLC plate was prepared with silica gel G (silica gel G f_{254}, thickness 0.25 mm). TLC was run by using benzene: n-butanol: acetic acid as solvent system (70:25:5) (Sridevi et al. 2008). The extracted sample and standard IAA (10mg/100ml) were spotted on TLC plate and observed for the development of chromatogram which was developed with the spray of Salkowski's reagent on TLC plate

(Kuang-Ren et al. 2003). Rf value of the standard and IAA produced by the isolate was calculated as-

$$\text{Rf value (refrective index)} = \frac{\text{Distance of solute travelled}}{\text{Distance of solvent travelled}}$$

iii. Characterization:

Extracted IAA sample was characterized by Fourier transmission infra-red (FTIR) spectroscopy to identify the chemical bonds and molecular structure (Kamnev et al. 2001; Goswami et al. 2014). FTIR analysis was carried out by FTIR spectroscopy (Thermofisher Scientific, NicoletTM6700) at USIC, BBA University, Lucknow. Methanolic extract of IAA was completely dried and mixed with spectral grade potassium bromide (KBr). FTIR spectral analysis was recorded at the transmission mode from 400–4000 cm^{-1} (Goswami et al. 2014).

3.5.8 EPS production:

For the estimation of EPS production bacterial isolates were inoculated into conical flask containing 20 ml of YEM broth media supplemented with 5% of carbon source (sucrose) (Mody et al. 1989). The inoculated flasks were incubated at 30 °C for 72 h in shaking condition. After incubation, broth was centrifuged (at 8000 rpm at 4 ºC for 10 minutes) and the supernatant was mixed with chilled acetone (96%) in 1:2 ratios. After adding acetone crude EPS was precipitated from supernatant which was collected by centrifugation for 30 min. Collected EPS was dried for half an hour of drying at 105 ºC and weighed (Damery and Alexander 1969).

i. Optimization:

Various growth parameters were optimized for EPS production by selected isolate. Growth parameters for optimization of EPS production were checked out various salt concentrations (1%, 2%, 3%, 4%, and 5%), pH (5, 6, 7, 8 and 9), carbon sources (glucose, galactose, dextrose, mannitol, mannose, lactose and inositol) and nitrogen sources (ammonium sulphate, ammonium chloride, sodium nitrate, yeast extract, glycine, and potassium nitrate). Carbon sources were added to the media at 1% level and nitrogen sources were added at 0.1% level (Ghosh et al. 2005). YEM broth was used for all the experiments and after inoculation incubated for 48 hours at 28 ºC. After the completion of incubation period OD was taken at 540 nm and compared.

ii. Composition analysis:

After optimization EPS was produced at optimized cultural conditions for further characterization. Estimation of extracted EPS was done by phenol-sulphuric acid method (Dubois et al. 1956). In this method EPS was taken in a tube and 1 ml of sterile water was added in it. Then 400 µl of EPS solution was taken in a test tube and 400 µl of phenol (5%) and 2 ml concentrated H_2SO_4 was added. After this the tubes were kept in water bath for 15-20 minutes at room temperature and OD was taken at 490 nm. EPS was estimated by comparing with standard curve of glucose.

Component analysis of EPS was also done by thin layer chromatography (Horborne 1976). Precipitated EPS sample was hydrolysed with 2 vol of H_2SO_4 (2.5 M) at 100 ºC for 1 hr. This solution was neutralized with sodium carbonate (1M) and spotted on silica gel plate (Merck 60F 254). The plate was developed in TLC chamber using n-butanol: acetic acid: water (4:1:5 v/v/v) as the mobile phase at room temperature. The plate was air dried, sprayed with alkaline potassium permanganate, and incubated at 100 °C for 10 min. The Rf values of the colored spots were measured and compared with standard carbohydrate (glucose).

iii. Characterization:

Characterization of EPS was done by SEM, energy dispersive spectroscopy (EDS) and FTIR techniques. SEM and EDS Analysis were done by scanning electron microscope

(JEOL, JSM6490LV). SEM was done for determining surface morphology of the EPS. FTIR analysis was done for molecular characterization (functional group analysis) of EPS (Thermofisher Scientific, NicoletTM6700) at USIC, BBAU, Lucknow. Extracted EPS was completely dried and mixed with spectral grade potassium bromide (KBr) and analysed at 500-4000 cm^{-1}.

3.5.9 Biocontrol activity against phytopathogens:

Antagonistic activity of the isolates against fungal phytopathogens (*Fusarium oxysporum, F. moniliforme* and *F. solani*) was done on YEMA plate by dual culture method (Arora et al. 2001). Phytopathogens were taken from Culture Collection of Rhizosphere Microbiology Laboratory, Department of Environmental Microbiology, BBA University, Lucknow (Uttar Pradesh, India). Fungal strains were grown on potato dextrose agar (PDA) at 28 ºC (Himedia, Mumbai) and also maintained on PDA plate at 4 ºC. A 6 mm mycelial disc of each fungal strain was excised from the edge of an actively growing antagonist and placed at the centre of a fresh YEMA plate. Exponentially grown cultures of each isolates were spot inoculated on either side of the fungal disc (equidistant from the periphery) and incubated at 28 ºC for 5 days. In the control plate, only mycelial disc of fungus was placed. After incubation, radial growth of fungus was measured. The percent inhibition of average radial growth was calculated relative to the control by following equation (Udomsilp et al. 2009):

$$L= C-T/C \times 100$$

Where L= percent inhibition of radial mycelial growth, C= radial growth of the pathogen in the control, T= radial growth of the pathogen in the presence of isolated strains.

3.6 Nodulation test of isolates:

Nodulation ability of all the isolates were checked on their respective hosts and also various common leguminous plants such as *Pisum sativum* L. (cow pea), *Cicer arietinum* L.

(chickpea), *Vigna radiata* L. (moong), *Vigna mungo* L. (urd), and *Trigonella foenum-graecum* L. (Methi). Nodulation assay was done in culture tubes (glass tubes) of 170 ml (38x200) capacity. Nitrogen free Jensen's seedling agar media was used for nodulation test (Jensen 1942). Culture tubes were filled with 70 ml sterile media (Engelke et al. 1987). All seeds were surface sterilized with with 0.2 % $HgCl_2$ for five minutes and then rinsed with 70% alcohol followed by water (5-6 times). Surface sterilized seeds were pre-germinated on sterilized water-agar (0.8%) and the seedlings were transferred in culture tubes (Gnat et al. 2015). After three days seedlings were inoculated with YEM broth cultures of bacteria (10^8 cells/ml) in three replicates. Non-inoculated plants were used as negative controls. Symbiotic affectivity of rhizobia was estimated based on their nodule formation and the dry weight of shoots of inoculated plants compared to the control. The medium containing portion of tubes was covered with black paper and tubes were incubated under controlled conditions. After three weeks the plants were harvested and examined for nodule development (Hardy et al. 1968).

Nodulation ability of isolates was also checked in small plastic pots (20x15) filled with 2 kg sterilized soil. Surface sterilized seeds were sown in the pots in 05 replicates. After seed germination seedlings were treated with fresh grown bacterial culture (10^8 cells/ml). After one week of seed germination similar booster dose of the same inoculum was also given. Plants were irrigated with sterilized water and after 60 days plants were carefully uprooted and observed for nodule formation and seedling biomass.

3.7 Genotypic characterization:

After phenotypic characterization, PGP characterization and nodulation test 11 selected isolates were identified at species level by the 16s RNA gene sequencing and diversity analysis of four selected rhizobial isolates was done by random amplified polymorphic DNA (RAPD) technique.

3.7.1 16S rRNA sequencing:

Bacterial isolates were cultivated on YEMA plates at 28 °C for 5 days and used for DNA extraction. DNA was isolated using standard protocols. After DNA extraction PCR amplification of the 16S rRNA gene was done by using primers; 27F (5´ AGAGTTTGATCMTGGCTCAG 3´) and 1492R (5´ CGGTTACCTTGTTACGACTT 3´). Amplification reactions were performed under following conditions:

PCR conditions:

Reaction Mixture (50 µl)		**Cycling Conditions**		
Template DNA	100 ng	Initial Denaturation	2 minutes at 95°C	
Forward Primer	0.3 µM	Denaturation	30 seconds at 95°C	35 Cycles
Reverse Primer	0.3 µM	Annealing	30 seconds at 52°C	
Master Mix	25 µl	Extension	2 minutes at 72°C	
Nuclease Free Water	Volume makeup 50 µl	Final Extension	15 minutes at 72°C	

Primer details for PCR:

S. No.	**Oligo name**	**Sequence (5`-3`)**	**Tm (°C)**	**GC- Content**
1	27F	AGAGTTTGATCMTGGCTCAG	56.3	47.5%
2	1492R	CGGTTACCTTGTTACGACTT	55.3	45%

On the basis of PCR results isolates showed a single fragment amplification which was used for the sequencing of the 16S rRNA gene from both strands using the primers; 785F (**5`** GGATTAGATACCCTGGTA **3`**) and 907R (**5`** CCGTCAATTCMTTTRAGTTT **3`**). Primer details are given below:

Primer details for sequencing:

S. No.	**Oligo name**	**Sequence (5`-3`)**	**Tm (°C)**	**GC- Content**
1	785F	GGATTAGATACCCTGGTA	56.3	47.5%
2	907R	CCGTCAATTCMTTTRAGTTT	55.3	45%

The amplified products were analysed by gel electrophoresis. The resulting amplicons (5 µl) of the 16S rRNA genes were mixed with loading buffer (2 µl) and analysed electrophoretically through 1 % agarose gel stained with ethidium bromide (10 mg/ml) at 100 V for 60 min. The bands were observed under UV light and image was captured.

3.7.2 Phylogenetic analysis:

The 16S rRNA gene sequences of the isolated strains and the sequence of the reference strains in GenBank were aligned using the software Molecular Evolutionary Genetics Analysis (MEGA, ver. 6.0) (Saitou and Nei 1987). Phylogenetic tree construction and evolutionary history were inferred according to the Neighbor-Joining method (Tamura et al. 2013). Similar identities of 16S rRNA gene sequences were also analysed with National Center for Biotechnology Information (NCBI)-BLASTn programme (www.ncbi.nlm.nih.gov/blast) (Altschul et al. 1990) and Ez-Taxon server (www.ezbiocloud.net/eztaxon) (Yoon et al. 2017).

3.7.3 Sequence submission to NCBI GenBank database:

The 16S rRNA gene sequences of the isolates were deposited in the NCBI GenBank database and accession numbers of each isolates were obtained.

3.7.4 DNA fingerprinting of isolates by RAPD technique:

Diversity analysis of rhizobial isolates (AB3, LB2, AB1 and VYS) was done by RAPD technique (Pongsilp et al. 2012). For template preparation, DNA was taken from the isolates grown in YEM broth for 2 days at 28 °C by phenol–chloroform method (Maloy 1990). Bacterial cells (2.4×10^6 cells) were harvested and centrifuged for 5 min at 300×g in a 1.5 ml eppendorf tube. Supernatant was removed completely and discarded without disturbing the cell pellet. After that cell pellet was again suspended in 200 µl lytic buffer and 5 µl of RNAse solution added (10 mg/ml) and incubated for 1 hour at 37 ºC. 2 µl of proteinase K (10 mg/ml) was added and incubated for 1 hour at 37 ºC. It was centrifuge at 12,000× g for

10 mins and supernatant was mixed with equal volume of phenol/chloroform/isoamyl alcohol, extracted thoroughly, and centrifuged at 10000 rpm for 5 minutes. For precipitation of nucleic acid 50µl of 3M sodium acetate and 1 ml of chilled ethanol was added and DNA precipitate was collected. This DNA sample was centrifuged (10,000 × g for 10 minutes) and washed in 70% ethanol and again centrifuged (10,000 × g for 10 minutes). After centrifugation supernatant was discarded and pellet was air dried and then dissolved in 200 µl TE buffer. PCR of collected DNA was done at following conditions:

PCR Conditions

Reaction Mixture (50 µl)		Cycling Conditions		
Template DNA	100 ng	Initial Denaturation	4 minutes at 95°C	
Primer	25 µM	Denaturation	1 minutes at 95°C	40 Cycles
		Annealing	1 minutes at 35°C	
Master Mix	25 µl	Extension	2 minutes at 72°C	
Nuclease Free Water	Volume makeup 50 µl	Final Extension	15 minutes at 72°C	

Primer Details

S. No.	Oligo name	Sequence (5`-3`)	Tm (°C)	GC- Content
1	OPA-16	**5`CACACTCCAG 3`**		

After PCR gel electrophoretic analysis of amplified products was done. In this method 5µl of DNA samples were resolved on 1% Agarose gel at 80V for 60 min and gel was visualized under UV light and the image was captured. Clustal analysis of gene sequence was calculated and made dendrograms using the unweighted pair group method with arithmetic mean clustering algorithm (UPGMA) with the help of PyElph 1.4 software (Pavel and Vasile 2012).

3.8 Calculation of biodiversity index:

Diversity index of all the isolates in respect to their host plant was calculated by online biodiversity calculator site https://bpmsg.com/diversity-online-calculator/ (Goepel 2012).

Diversity index of selected isolates was also calculated after their genotypic characterization (Goepel 2012).

3.9 Application of isolates on their respective host:

Among all some isolates were selected on the basis of their PGP activities and applied on their respective host (*Abrus*, *Clitoria*, *Crotalaria*, *Leucaena* and *Sesbania*) to check their impact on plant growth both in laboratory conditions and pot assay.

3.9.1 *In vitro* application:

In laboratory conditions impact of isolate on growth of their host plant was checked in culture tubes by using water ager media. Before treatment with bacterial culture all types of seeds were surface sterilized with 70% ethanol (2 min) and 2% sodium hypochlorite (10 min) followed by 7-8 times washings with sterilized distilled water. For seed treatment bacterial inocula was prepared by growing bacterial isolates in YEM broth medium at 28 ºC in incubator shaker (180 rpm) for 48 hrs. Inoculum of each bacterial culture was prepared in separate flasks. After incubation the OD of each broth was adjusted to 01 at 600 nm (Bharti et al. 2013). Aqueous solution (1%) of corboxymethylcellulose (CMC) was also added with bacterial cultures as binding agent (4 ml CMC with 1 g seed) (Waller and Cook 1986). Surface sterilized seeds were mixed with CMC solution and respective bacterial cultures, kept overnight and air dried for 2 hr. After treatment uniform sized seeds were placed in culture tubes (3 in each) at suitable growth conditions and observed for their germination and growth. Seeds without bacterial treatment were used as control. Control of each type of seeds was placed in separate tubes. Seed germination rate and various growth parameters such as root length, shoot length, leaf count, fresh weight and dry weight of all plants were observed after 15 days. Chlorophyll content was also measured by standard protocols according to Arnon (1949).

3.9.2 *In vivo* application:

Pot experiment was conducted in plastic pots (24x12x12 cm) filled with autoclaved soil with 3 replicates. The physicochemical properties of experimental soil were analyzed by standard protocols (Chapman and Pratt 1961) and formed as pH-8.2, electrical conductivity-3.5ds/m, water holding capacity-55%, organic carbon-0.34%, total nitrogen-973.45Kg/H, total phosphate-11.23Kg/H, soluble potassium-210.34Kg/H and organic matter-0.61%. For this experiment surface sterilized seeds of each legumes were treated same as *in vitro* application. Treated seeds and uninoculated control seeds were sown in pots (5 in each) and observed for germination and growth. After 30 days of sowing plants were uprooted carefully and various growth parameters of all plants such as root length, shoot length, leaves count, fresh weight and dry weight were observed and analysed. Various nutritional contents of all plants were also measured by standard methods.

3.9.3 Analysis of nutritional contents of plants:

Various types of nutrient contents of all the plant samples were also determined by standard methods which are given below:

i. Nitrate estimation:

This was done by method of Cataldo et al. (1975). In this method 5% salicylic acid and 2N sodium hydroxide (NaOH) solution was used as reagents for nitrate estimation. Potassium nitrate was used as standard.

ii. Carbohydrate estimation:

This was done by phenol-sulphuric acid method using 5% phenol and 96% sulphuric acid (reagent grade) to estimate total carbohydrate in plant extract by taking glucose as standard (Dubois et al. 1956).

iii. Protein estimation:

Protein estimation was done by Bradford reagent (Marion and Bradford 1976). Bradford reagent contains Coomassie Brilliant Blue G-250 dye as main component and bovine serum albumin (BSA) was used as standard to calculate protein content.

iv. Chlorophyll estimation:

Chlorophyll (a, b) content of leaves was estimated by Arnon (1949) method in which 80% acetone was used. Chlorophyll was estimated by taking optical density at 663 nm and 645 nm and acetone used as control.

v. Antioxidant activity:

Antioxidant activity of extracted plant sample from each treatment was done by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as radical scavenging agent (Braca et al. 2001). In this assay extracts of plant leaves were added to 3 ml of 0.004% methanol solution of DPPH and absorbance was measured at 517 nm for estimating total antioxidant activity.

v. Flavonoid content:

The flavonoid content in the leaves extracts was determined by spectropboiledometric method (Quettier et al. 2000). The plant sample (aqueous solution of the extract in the concentration of 1mg/ml) and 1ml of 2% aluminium chloride ($AlCl_3$) solution (methanol) was mixed and incubated for an hour at room temperature. The absorbance was measured at wavelength of 415 nm and flavonoid activity calculated.

3.10 Statistical analysis

All the data of plant growth parameters was analysed statistically by one way analysis of variance (ANOVA) and Duncan’s multiple range test (DMRT) at 5% level of significance to compare difference between treatment means (Gomez and Gomez 1984). Statistical analysis was done by software statistical package for the social science (SPSS) (2016) for windows.

3.11 Submission of useful strains in culture collection centres:

Selected bacterial isolates (AB3, LB2 and CV2) were submitted as type strains in various culture collection centres as general deposit (Sharma 2014). Submitted culture was assigned with accession number by collection authority. Culture submission in American Type Culture Collection (ATCC) is also in progress. Submission was done in following culture collection centres-

i. Microbial Culture Collection (MCC), Pune
ii. Microbial Type Culture Collection (MTCC), Chandigarh
iii. National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau

RESULTS

4.1 Bacterial isolates and their morphological properties:

In this study 40 root nodulating bacteria were isolated from root nodules of five wild medicinal legumes (*Abrus, Clitoria, Crotalaria, Leucaena* and *Sesbania*) collected from different sites (**Fig. 7, 8 & Table 8**). All the isolates formed convex, mucilaginous colonies with smooth margins (2 to 4 mm diameter) on YEMA media plates (**Fig. 10, 11**). Sixty percent of the isolates formed white colonies and remaining 40% formed beige pigmented colonies. Isolates were also grown on CRYEMA media and it was observed that most of the isolates did not absorb red colour, forming white slimy colonies and some isolates were able to absorb red colour at a very fast rate and few isolates absorbed at very slow rate. All the isolates were Gram negative and rod shaped in nature (**Fig. 12**). All the isolates showed positive motility in motility test agar media (**Fig. 13**). In SEM analysis shape of bacterial isolates were found to be rod shaped and medium sized bacteria. Size of isolates ranged from 1.50-1.75 µm in length and 0.50-0.58 µm in width (**Fig. 14**).

4.2 Analysis of internal structure of root nodules:

Root nodules of collected plants (**Fig. 15**) were analysed for their internal structure. In simple staining section of root nodule was observed with various structural components such as cortex layer (inner and outer), symbiotic zone with various cells infected with rhizobia and few cells without rhizobia were also seen (**Fig. 16**). Similar structure was also observed under electron microscopy (**Fig. 17**).

4.3 Physiological and metabolic properties of isolates:

Most of the isolates (75%) were able to produce acid by utilizing glucose and showed yellow coloration around the mucilaginous colonies and remaining 25% isolates were less mucilaginous and produced alkali as indicated by the production blue colour on BTB supplemented YEMA media plate. Generation time of fast growing isolates ranged from 2.5-3.5 hrs while generation time of slow growers ranged from 7.0-7.5 hrs (Table 8).

Physiological properties of isolates were checked by analysing their growth on various cultural conditions. All the isolates were able to utilized most of the applied carbon sources such as mannitol, glucose, dextrose, lactose, galactose, sucrose and maltose. Other carbon sources such as starch, glycerol, fructose, citrate, malate and trehalose were also utilized by the isolates (Table 9). All the isolates were also able to utilize most of the tested nitrogen sources such as yeast extract, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, glutamine and tryptophan. None of the isolates were able to utilize glycine while most of the isolates were able to utilized amino acids such as cysteine, lysine and methionine as nitrogen sources (Table 10). On the basis of this it can be confirmed that most of the isolates were able to utilize diverse range of carbon and nitrogen sources for their growth.

Optimum temperature for the growth of isolates was 28 ºC but they were also able to grow at temperatures ranging from 15-35 ºC. All the isolates were able to grow upto 4% salt concentration and 42.5 % isolates were able to grow upto 8% salinity level in growth media. Optimum pH level for the growth of isolates was 7.0 but some of the isolates were able to grow from pH 4 to 12. All the isolates were able to grow at pH 6-10. However 27.5% isolates were also able to grow at pH 4 to 12 (Table 11).

The analysis of the intrinsic bacterial resistance to antibiotics showed that isolates were sensitive to all the tested antibiotics except penicillin (**Fig. 18**). All the isolates were sensitive for most of the antibiotics but sensitivity index of isolates were recorded with very diverse values for each antibiotic. Only two isolates (SB2 and KSI) were resistant for ampicillin and another two isolates (CB2 and SB1) were also resistant for erythromycin (Table 12).

4.4 Biochemical properties of isolates:

All the forty isolates were positive for catalase activity by liberating oxygen on addition of hydrogen peroxide on the colonies. All the isolates showed positive oxidase, nitrate reductase and ammonia production ability. None of the isolates showed positive gelatinase and cellulase activity. Most of the isolates 62.5% and 72.5% showed growth on GPA media and potassium nitrate (8%) supplemented media, respectively. Among all the isolates 27.5% were able to grow on HAM media and showed positive lactose test by forming yellow colour zone around the colonies. Most of the isolates (80%) were positive for citrate utilization by forming blue coloration around the colonies. Among all the isolates 57.5% isolates were positive for urease activity, 62.5% for MR test and 37.5% for VP test. Most of the isolates (77.5%) were able to produce PHB which was confirmed by formation of black precipitation on bacterial colonies on addition of sudan black dye. Enzymatic activities such as amylase, protease and lipase were also showed by 67.5% 42.5% and 2.5% isolates, respectively. On the basis of these biochemical tests all the isolates were observed to be very diverse type (**Fig. 19 & Table 13**).

4.5 Plant growth promoting properties of isolates:

4.5.1 Nitrogen fixation:

Among all the isolates 97.57% showed nitrogen fixing ability which was observed by their growth on nitrogen free media plate. Except isolate SN all the isolates showed nitrogen fixing ability but five isolates (AB3, ClU1, LM, LB2 and VYS) showed higher ability to fix nitrogen in comparison to others (**Fig. 20 & Table 14**).

4.5.2 Phosphate solubilisation:

Phosphate solubilisation was shown by 80% of the isolates. Among all the isolates AB3 showed maximum phosphate solubilisation ability with 2.3 psi by forming maximum clear zone with 2.3 cm diameter. Isolate SN showed the least solubilisation index 0.7 psi (**Fig. 20 & Table 14)**.

4.5.3 Zinc solubilisation:

Among all the forty isolates, 70% were able to solubilize zinc as observed by clear zone formation around the colonies. Isolate LB2 showed maximum zinc solubilisation ability (2.6 psi) followed by isolate AB3 with 2.5 zsi while least solubilisation was shown by isolate SN with 0.6 zsi (**Fig. 20 & Table 14)**.

4.5.4 HCN production:

Only one isolates (2.5%) SM1 was able to produce HCN as observed by colour change of filter paper soaked in picric acid from yellow to orange (**Fig. 20 & Table 14)**.

4.5.5 Siderophore production:

Siderophore production was showed by 50% isolates, forming orange coloured zone around the bacterial colonies in qualitative analysis on CAS agar plates. LB2 showed highest siderophore production ability with larger orange zone on CAS plate. Quantitatively LB2 showed highest siderophore production ability with 45.12 psu and 45.64 psu by traditional and microplate method respectively (**Fig. 20 & Table 14)**.

4.5.6 Gibberellin production:

Among all the forty isolates 60% isolates were able to produce gibberellin. Isolate ClU1 showed highest gibberellin production with 3.0µg/ml and SB1 showed least production with 0.9 µg/ml (**Fig. 20 & Table 4.5)**.

4.5.7 IAA production:

IAA production ability was showed by 60% isolates which was observed on the basis of spectrophotometric analysis and colour change. AB3 was identified as a very potential IAA producer. AB3 produced maximum amount of IAA with value 120 µg/ml. At optimum cultural conditions IAA producing ability of AB3 was maximum (**Graph 1**). It was found that AB3 showed best IAA producing ability when growth medium was supplemented with 0.2% tryptophan, 0.5% salt concentration, mannitol (1%) as carbon source, potassium nitrate (0.1%) as nitrogen source at neutral pH and 72 hrs of incubation. At these optimized growth conditions IAA was produced and extracted in powder form. Pure IAA was extracted from bacterial culture by solvent extraction method in ethyl acetate layer. Extracted IAA was purified by TLC and chromatogram of culture showed a pink spot of purified IAA at the Rf value (0.65) almost same to standard IAA (0.67) (Fig. 24). Extracted IAA sample was further characterized at molecular level by Fourier transform infrared (FTIR) spectroscopy and characteristic indole group was observed (Fig. 25). In the FTIR spectra peak observed at wavelength 3286.2 cm^{-1} showed the characteristic N-H stretching band of indole moiety. This is the identifying character of IAA. In spectra N–H bending was observed at wavelength 1640.6 cm^{-1}. Peak observed at wavelength 2924.7 cm^{-1} and 1460.6 cm^{-1} showed the presence of alkyl (–CH2) asymmetric stretching, symmetric stretching, and bending was observed respectively. FTIR spectra confirmed the presence of IAA (**Fig. 20 & Table 14)**.

4.5.8 EPS production:

All the isolates were able to produce EPS and AB3 was identified as highest EPS producer with value 75 µg/ml. AB3 produced maximum amount of EPS when the media was supplemented with mannitol (1%) as a carbon source and yeast extract (0.1%) as a nitrogen source at 3 % salt concentration at slightly alkaline pH-8 (**Graph 2**). Extracted EPS was solid white powder and soluble in water. On the basis of SEM analysis, EPS was observed as compact crystalline with irregular shape and rough surface (Fig. 26**)**. On the basis of result obtained by EDS it was observed that the concentration of calcium, phosphorus, potassium were highest in extracted EPS followed by oxygen and carbon. Magnesium and sulphur were also present in trace amounts. In this analysis EPS was observed as heteropolysaccharide with relative content of proteins and amino sugars having hydroxyl, carboxyl and amino as main functional groups. The recorded FTIR spectrum showed the characteristic peaks at various wavelengths (3204, 2923, 1672, and 1029 cm-^{1}) (Fig. 27). The band at 2923-3586 cm^{-1} was that of stretching vibration of hydroxyl group (O-H) which showed the EPS property and water solubility. The peak observed at 2923 cm-^{1} proved the presence of asymmetric vibration of C-H aliphatic group such as CH_2 and CH_3 groups. The peak observed at 1672 cm-^{1} showed the C-O stretching bands indicating the presence of gucosamine, succinate and acetate functions. Band observed around the 1322-1438 cm-^{1} could be for carboxylate groups (-COO-) from acid residues such as succinate groups. Finally the peaks around 1029 cm-^{1} indicated the presence of glucose as carbohydrate. Thus in this study most of the characteristic bands assigned by FT-IR spectroscopy indicate the succinoglycan type of EPS produced by AB3 isolate (**Fig. 20 & Table 14)**.

4.5.9 Biocontrol activity against phytopathogens:

It was observed that amongst all isolates, 35% (14 isolates) showed inhibition against *F. moniliforme*, 15% (6 isolates) against *F. solani* and 10% (4 isolates) against *F. oxysporum*. In case of *F. moniliforme* maximum inhibition was shown by isolate AB3 with 60.12% radial growth inhibition of fungus on YEMA plates. Maximum inhibition of *F. solani* was done by isolate LB2 with 55.34% radial growth of fungus on YEMA plates, while in case of *F. oxysporum* LB2 showed maximum radial growth inhibition of fungus with 48.43% in comparison to control. Isolate LB2 was most effective among all the isolates against tested phytopathogens followed by AB3 (**Fig. 22 & Table 15**).

4.6 Nodulation test of isolates:

Nodulation test was done to test the authenticity of isolates as root nodulating bacteria or rhizobia. Rhizobial isolates AB3, LM and VYS showed development of small pink nodules on their respective host *Abrus*, *Leucaena* and *Sesbania* in culture tube under laboratory conditions (**Fig. 28**). These three isolates (AB3, LM and VYS) were also able to nodulate their respective host in pot assay (**Table 16**). Isolates were not able to nodulate the legumes other than their host plant i.e. cross inoculation were not observed in this experiment (Fig. 29). AB3, LM and VYS were also enhanced with 40.29%, 60.00% and 55.55% increment in seedling biomass with nodule formation in comparison to uninoculated control.

4.7 Genotypic characterization of isolates:

4.7.1 16S rRNA sequencing and phylogenetic analysis:

By the analysis of 16S rRNA gene sequencing (**Fig. 30**) and nucleotide homology 11 best PGP isolates were characterized at genotypic level. On the basis of phylogenetic analysis these isolates were identified as strains from different genera. The sequence data of all the isolates were submitted to GenBank (NCBI) and assigned with respective accession numbers (**Table 17**). Among all (11) 5 isolates (AB1, AB3, LM, LB2 and VYS) were found to be of rhizobia group (order Rhizobiales), while 6 isolates (ClU1, ClU2, CV2, CV5, CGJ and LN) belonged to non-rhizobia group (order Enterobacteriales). Among 5 rhizobial isolates AB3 (**Fig. 31**), LM (**Fig. 32**) and VYS (**Fig. 35**) were identified as *Rhizobium pusense*, LB2 as *Rhizobium radiobacter* (**Fig. 34**) and AB1 as *Beijerinckia fluminensis (***Fig. 33***).* Out of 6 non-rhizobial isolates, 2 belonged to genus *Kosakonia* (ClU1 as *K. sacchari* and LN as K. *pseudosacchari*), 2 strains belonged to genus *Enterobacter* (ClU2 and CV5 as *E. cloaceae*), one strain CGJ belonged to *Cronobacter sakazakii* and one strain CV2 was *Pantoea agglomerans.* Among all the 5 wild medicinal legumes rhizobia were isolated from only 3 legumes (*Abrus*, *Leucaena* and *Sesbania*) and no rhizobia were isolated from root nodules of *Clitoria* and *Crotalaria*.

4.7.2 DNA fingerprinting by RAPD analysis:

Among all the identified strains, diversity of selected rhizobial strains (AB1, AB3, LB2 and VYS) were checked by RAPD technique. The data of RAPD showed considerable diversity in root nodulating bacteria of wild medicinal legumes (**Fig. 36**). On the basis of dendogram of RAPD analysis these rhizobial isolates are of three types. AB1, VYS and AB3 were belonged to same category and among these and LB2 showed higher diversity than other rhizobial isolates (**Fig. 37**).

4.8 Calculation of diversity indices among isolates:

Diversity indices among isolates in respect to their host plant were calculated as 4.0 with species richness value 5.0, which indicate high diversity level (**Table 18**). This diversity index also showed the diversity of wild medicinal legumes collected from different sites. Diversity indices among all the 11 genetically identified strains was calculated as 5.2 with species richness value 6.0, which also indicates the high diversity level among isolates (**Table 19**). Diversity indices among the rhizobial isolates was also calculated and recorded as 2.6 with species richness value 3. This value indicates the moderate type of diversity among isolates (**Table 20**).

4.9 Application of isolates on their respective host:

4.9.1 *Abrus:*

Isolate AB1 and AB3 were applied on their host plant *Abrus* and observed that AB3 was better plant growth promoter in comparison to AB1. AB3 caused higher growth in plants than control and AB1 (**Fig. 38 & 39**). It was observed that germination rate of AB3 treated seeds was 26.42% higher than uninoculated seeds. It was also observed that in comparison to control root length, shoot length and leaves count of AB3 treated plants significantly increased by 38.05%, 18.70% and 99.88% respectively. Fresh weight and dry weight of AB3 treated plants were also higher by 40.29% and 83.33% respectively, in comparison to control plants (**Table 21**). Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of AB3 treated plants were also significantly higher (by 14.61%, 6.97%, 16.93%, 13.73%, 195.4% and 50.00% respectively) in comparison to uninoculated plants (Fig. 40 & **Table 22**).

4.9.2 *Clitoria:*

Isolate ClU1 and ClU2 were applied on their host plant *Clitoria* and observed that ClU2 was better plant growth promoter than ClU1 (**Fig. 41 & 42**). ClU2 caused higher growth in plants in comparison to control and ClU1. It was observed that germination rate of ClU2 treated seeds was 11.76% higher than uninoculated seeds. It was observed that in comparison to control, root length, shoot length and leaves count of ClU2 treated plants significantly increased by 41.07%, 30.05% and 136.42%, respectively. Fresh weight and dry weight of ClU2 treated plants were also higher by 80.41% and 84.07% respectively, than control plants (**Table 23**). Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of ClU2 treated plants were also significantly higher by 18.66%, 6.28%, 16.24%, 15.84%, 136.00% and 45.16% respectively, in comparison to uninoculated plants (**Table 24**).

4.9.3 *Crotalaria:*

Isolate CV2, CV5 and CGJ were applied on their host plant *Crotalaria* and it was observed that among these 3 isolates CGJ was best plant growth promoter which caused highest growth in plant in comparison to control and other two isolates (CV2 and CV5) (**Fig. 43 & 44**). It was observed that germination rate of CGJ inoculated plant was 19.20% higher than uninoculated seeds. It was also observed that in comparison to control plants root length, shoot length and leaves count of CGJ treated plants were significantly increased by 43.29%, 17.54% and 81.33% respectively. Fresh weight and dry weight of CGJ treated plants were also higher by 123.07% and 123.30% respectively, than control plants (**Table 25**). Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of CGJ treated plants were also significantly higher by 15.62%, 05.01%, 23.80%, 16.61%, 245.45% and 38.70% respectively, in comparison to uninoculated plants (**Table 26**).

4.9.4 *Leucaena:*

Isolate LM, LN and LB2 were applied on their host plant *Leucaena* and it was observed that among these 3 isolates LB2 was observed as best plant growth promoter which caused highest growth in plants in comparison to control and other two isolates LM and LN (**Fig. 45 & 46**). It was observed that germination rate of LB2 inoculated plant was 15.25% higher than uninoculated seeds. It was also observed that in comparison to control plants root length, shoot length and leaves count of LB2 treated plants significantly increased by 69.59%, 31.64% and 52.68% respectively. Fresh weight and dry weight of LB2 treated plants were also higher by 90.00% and 70.32% with respectively than control plants (**Table 27**). Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of LB2 treated plants were also significantly higher by 25.47%, 05.25%, 16.53%, 14.91%, 179.16% and 53.33% respectively in comparison to uninoculated plants (**Table 28**).

4.9.5 *Sesbania:*

Isolate VYS was applied on its host plant *Sesbania* and observed as an efficient plant growth promoter which caused significant enhancement in growth of plant in comparison to control (**Fig. 47**). It was observed that germination rate of VYS inoculated plant was 4.18% higher than uninoculated seeds. It was observed that in comparison to control root length, shoot length and leaves count of VYS treated plants significantly increased by 55.86%, 16.75% and 32.25% respectively. Fresh weight and dry weight of VYS treated plants were also higher by 55.55% and 103.88% respectively, than control plants (**Table 29**). Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of VYS treated plants were also significantly higher (by 04.82%, 05.09%, 09.19%, 15.90%, 158.3% and 36.66% respectively) in comparison to uninoculated plants (**Table 30**).

4.10 Submission of useful strains in culture collection centres:

Amongst all the strains three best strains (AB3, LB2 and CV2) were submitted in culture collection centres (MCC, Pune, MTCC, Chandigarh and NBAIM, Mau). Culture submission in ATCC is in progress. Isolate **AB3** isolated from ***Abrus*** was confirmed as type strain of ***R. pusense*** and assigned with an accession number **MCC 3409** by MCC Pune, India (**Fig. 48**).

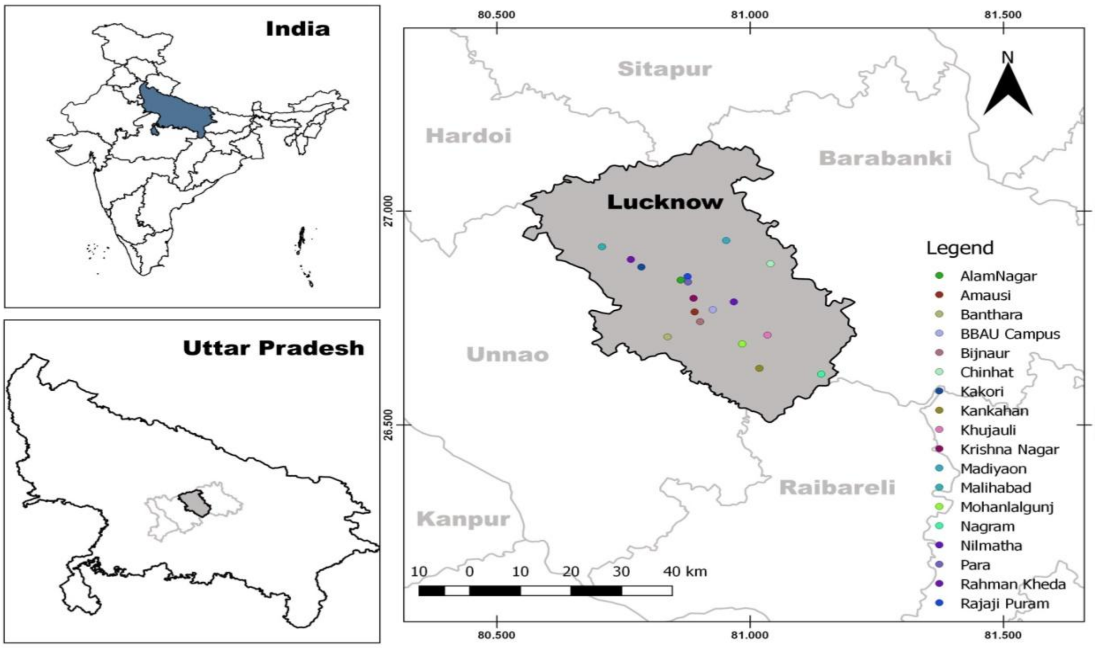


Fig. 8: Sampling sites

a) *Abrus* and root nodules b) *Crotalaria* and root nodules

c) *Clitoria* and root nodules d) *Sesbania* and root nodules

e) *Leucaena* and root nodules

Fig. 9: Collected wild medicinal legumes from different regions of Lucknow and adjoining areas

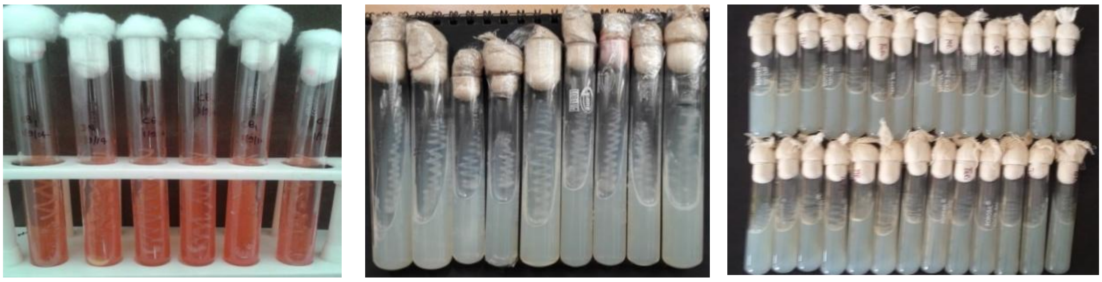


Fig. 10: Pure culture of bacterial isolates on YEMA/CRYEMA media slants

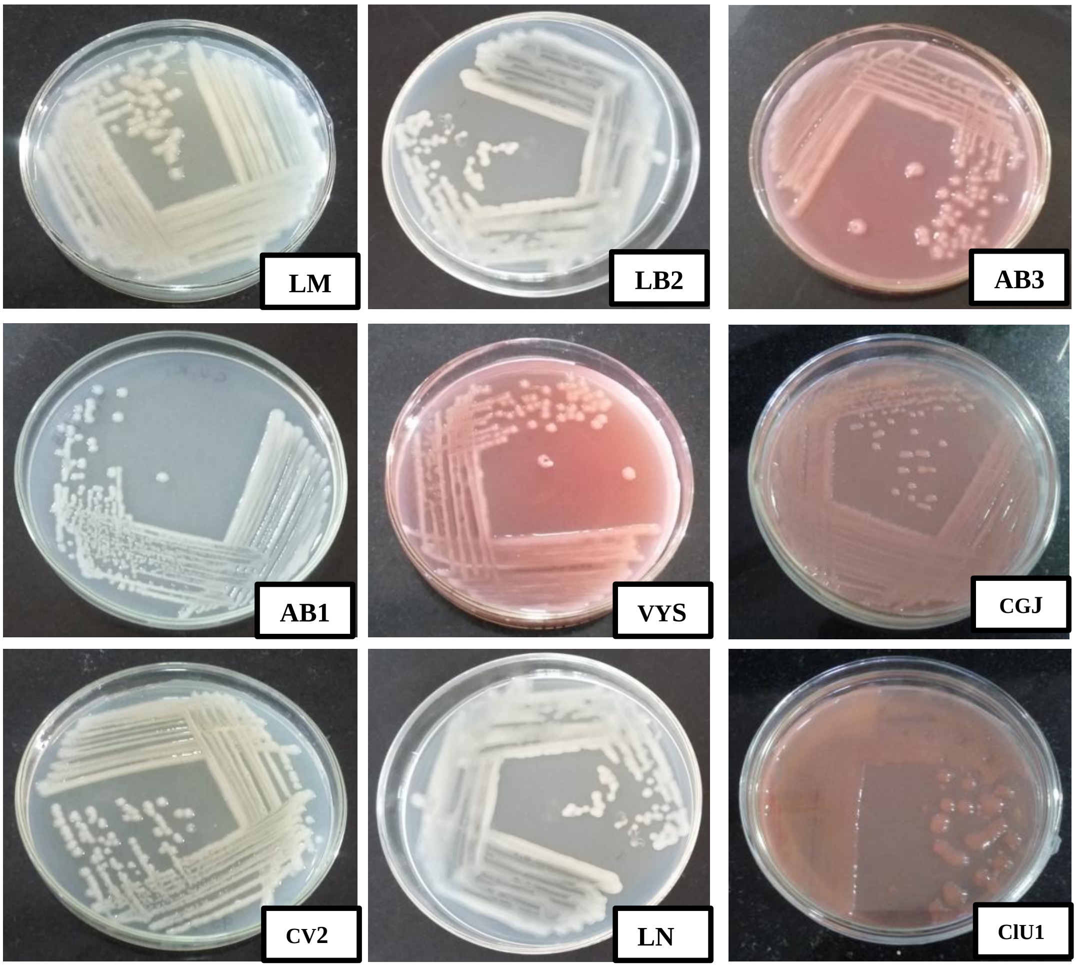


Fig. 11: Pure culture of bacterial isolates on YEMA/CRYEMA plates

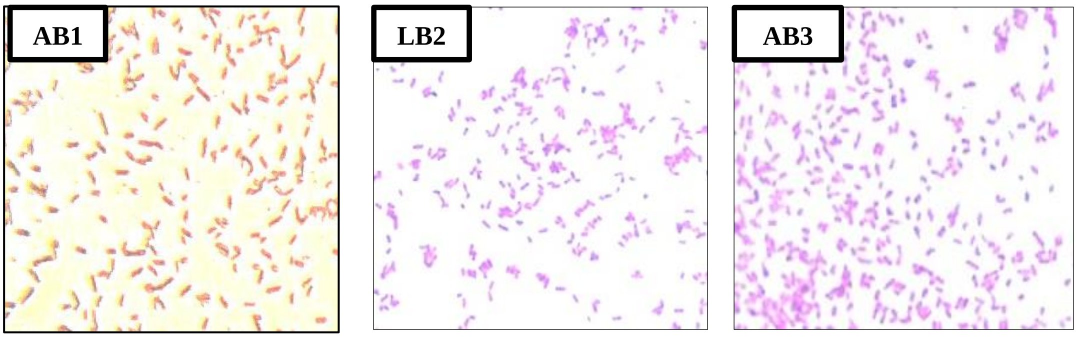


Fig. 12: Gram staining of isolate

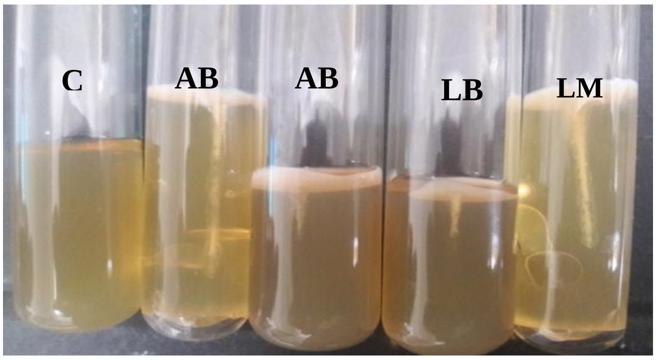


Fig. 13: Motility test of isolates

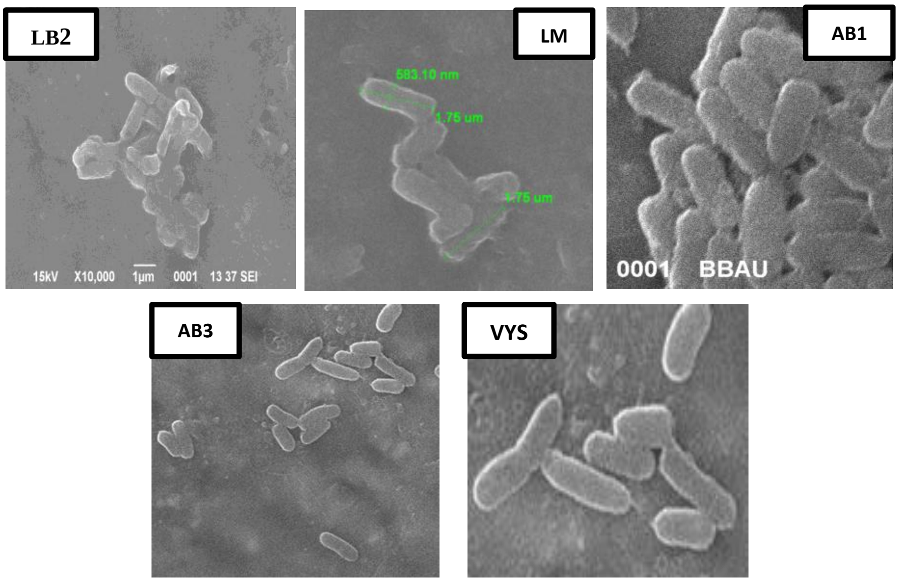


Fig. 14: Scanning electron micrograph of rhizobial isolates

Fig. 15: Root nodules of collected wild medicinal legumes from different regions

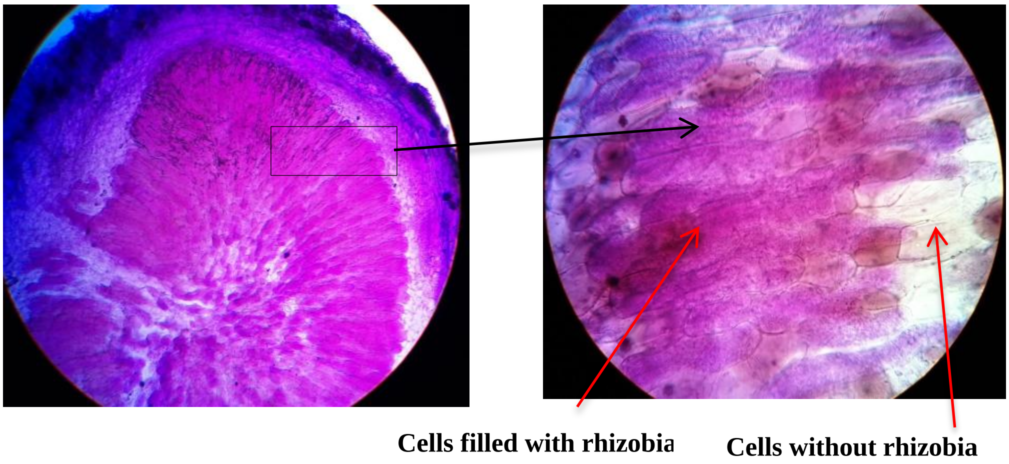
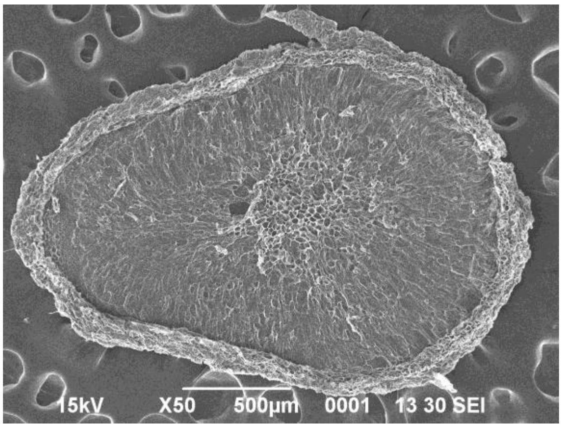
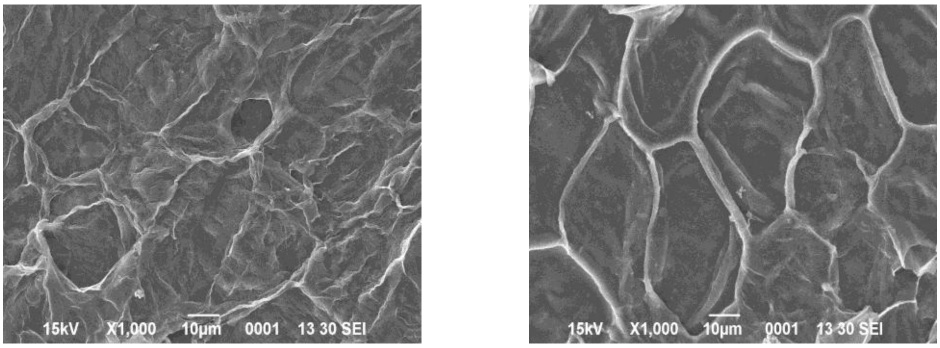


Fig. 16: Section of root nodules of *Abrus* (simple staining)



Section of root nodule



Root nodule cells filled with rhizobia Root nodules without rhizobia

Fig. 17: Internal structure of root nodule of *Abrus*

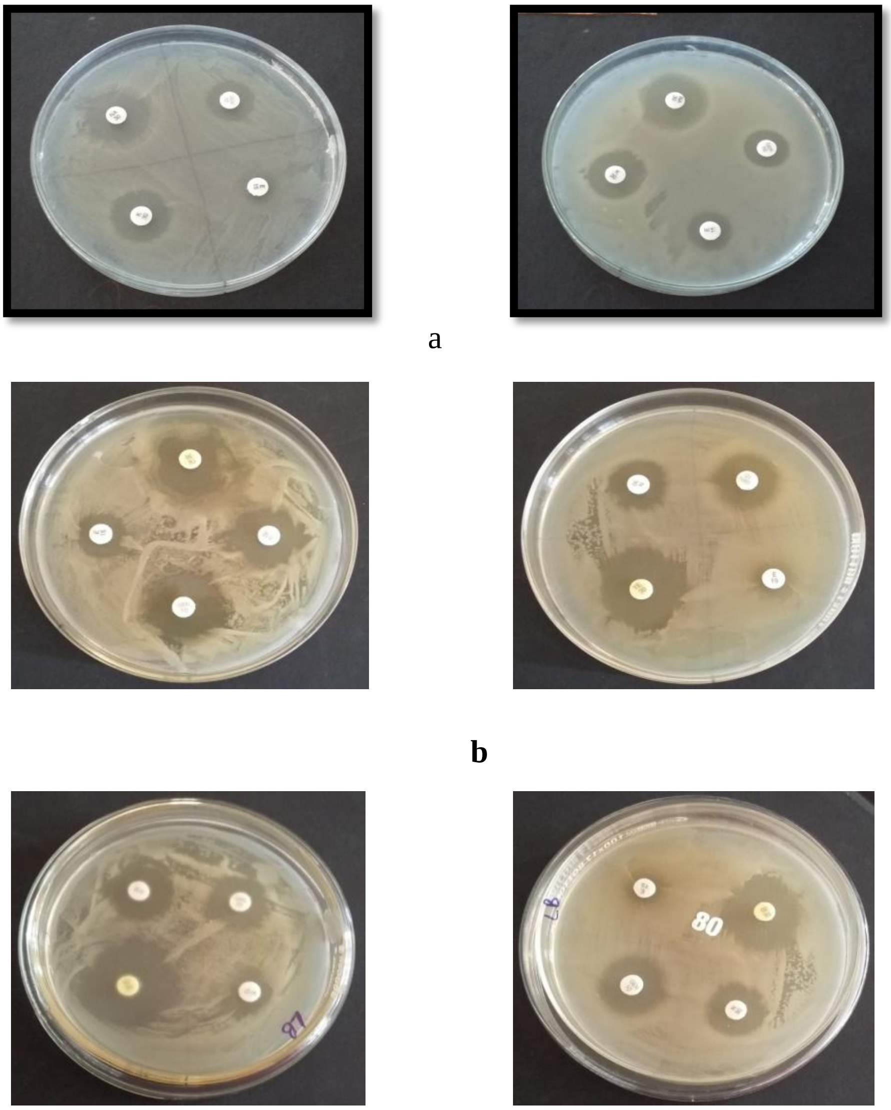
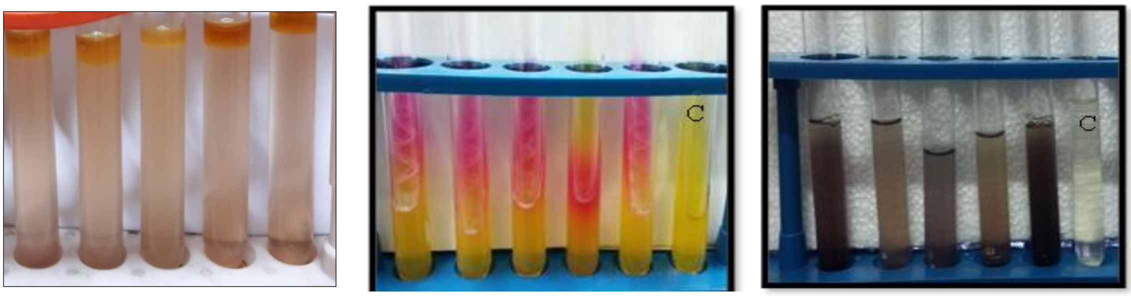
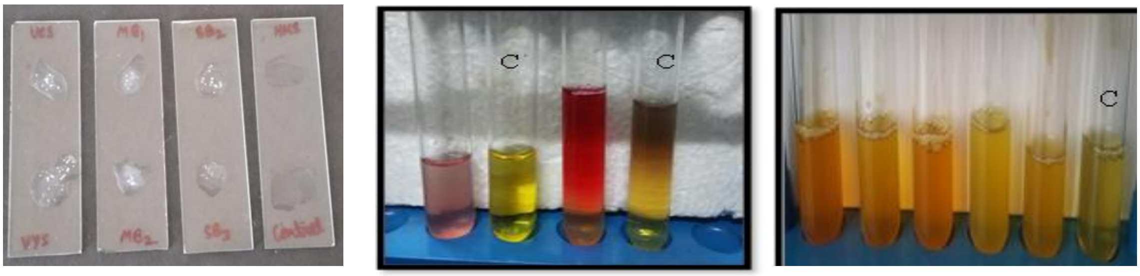


Fig. 18: Intrinsic antibiotic resistance activity of isolates a) AB1, b) AB3, c) LB2

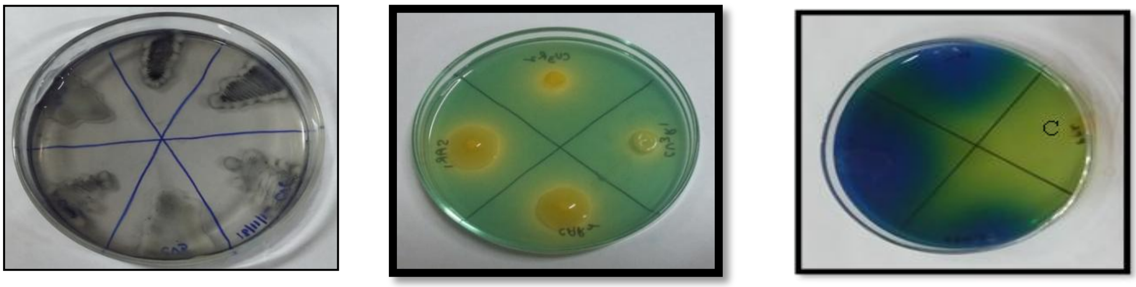


Indole test Urease test Nitrate reductase test

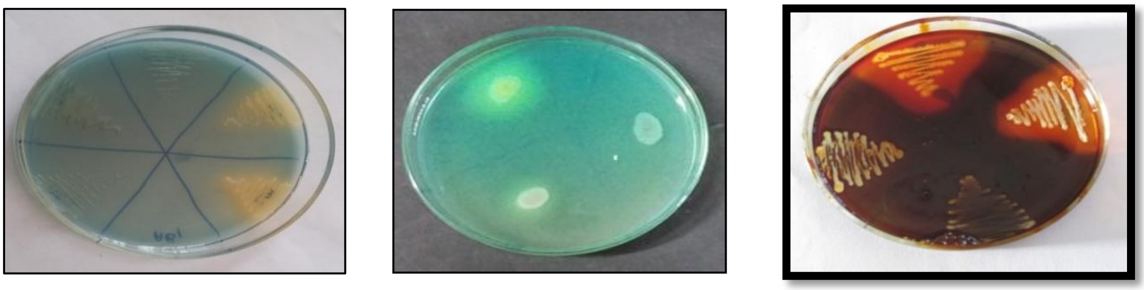


Catalase test MR-VP test Ammonia production

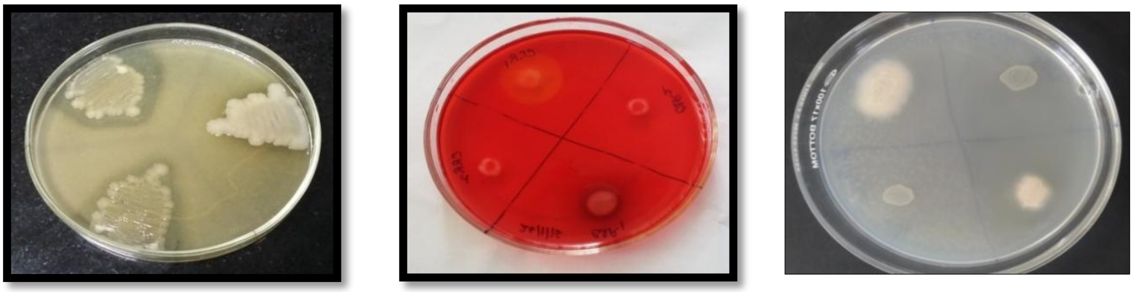
j



PHB production BTB test Citrate utilization



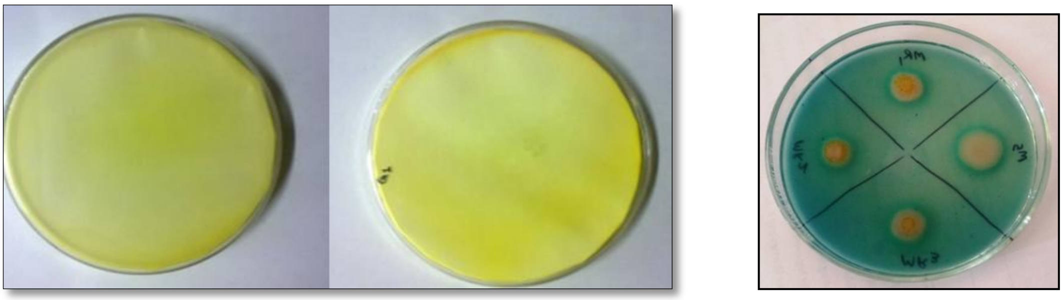
Growth on HAM Lactose test Amylase test



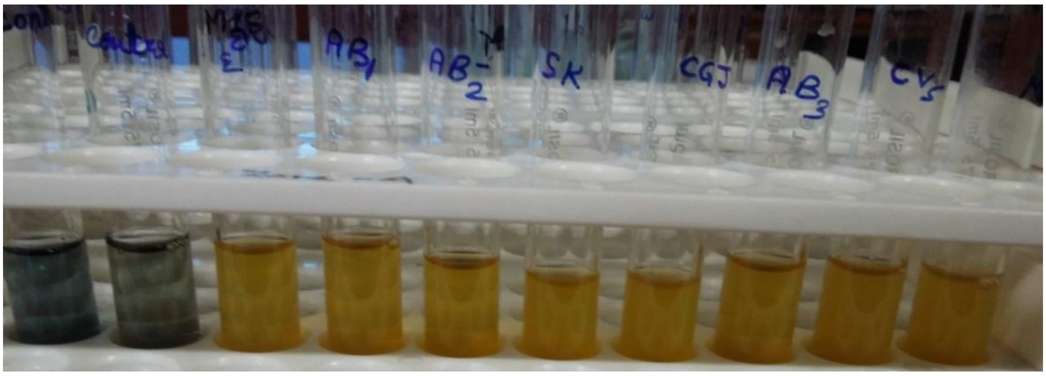
Protease test Cellulase test Lipase test

Fig. 19: Results of biochemical tests of isolates

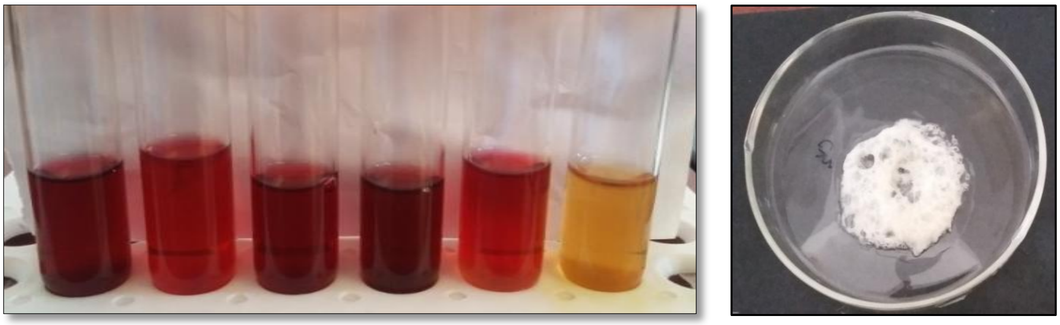
a) Nitrogen fixation b) phosphate solubilisation c) zinc solubilisation



d) HCN production e) siderophore production

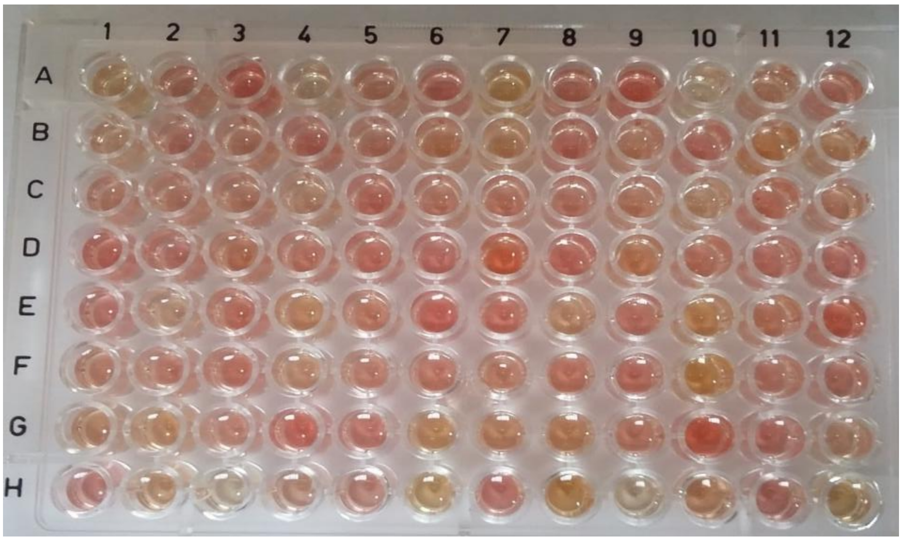


e) Siderophore production (quantitative)

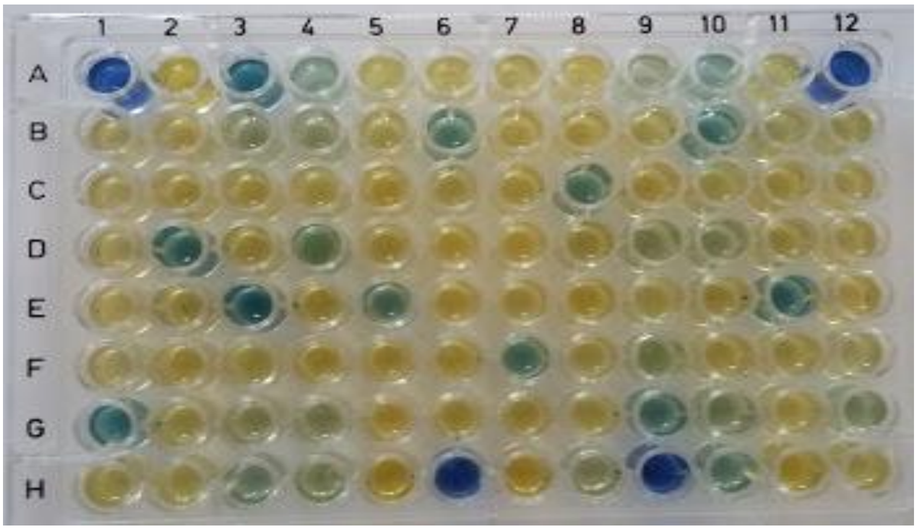


f) IAA production g) EPS production

Fig. 20: Plant growth promoting characters of isolates



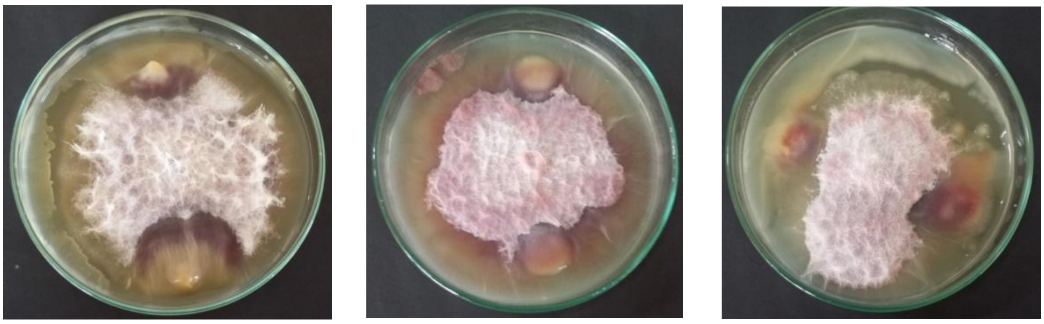
(a)



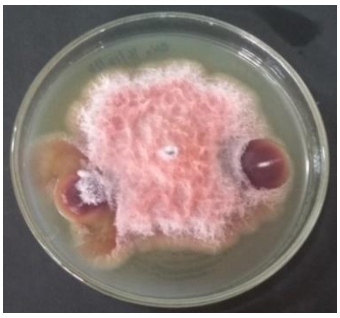
(b)

Fig. 21: Quantitative estimation of a) IAA production and b) siderophore production by isolates according to modified microplate method (Arora and Verma 2017)

Control LB2 AB3



ClU1 LB2 LM



VYS

Fig. 22: Biocontrol activity of isolates against *F. moniliforme*

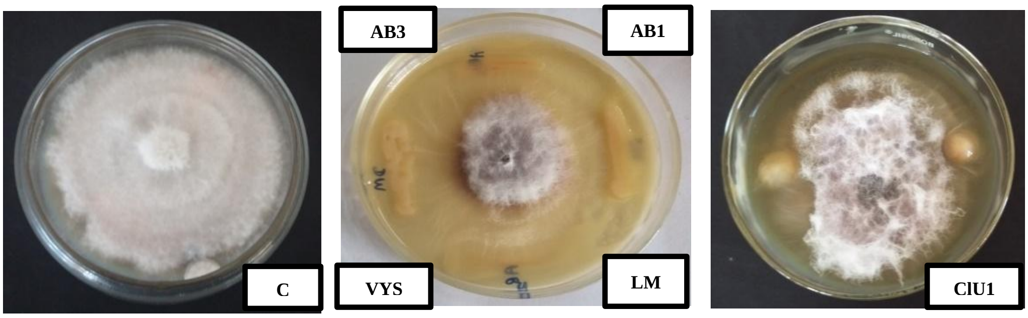


Fig. 23: Biocontrol activity of isolates against *F. solani*

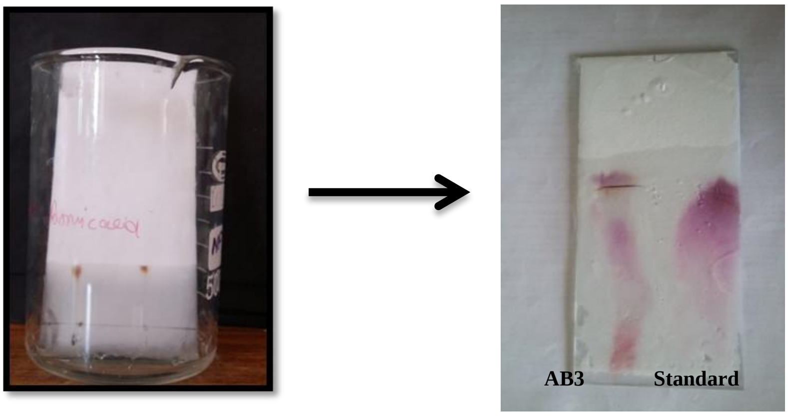
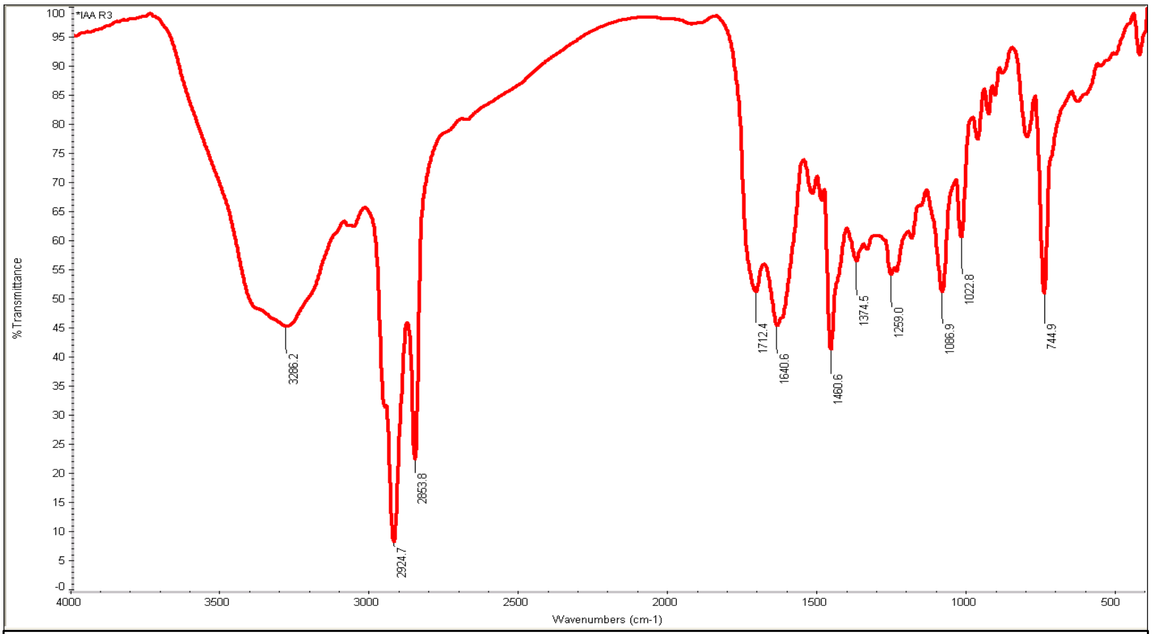


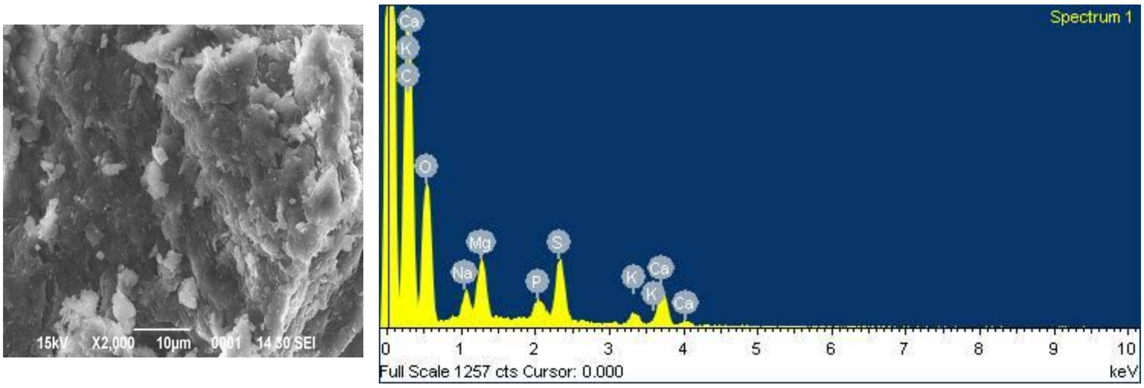
Fig. 24: Purification of extracted IAA sample by TLC



3286.2 - - Strong O-H, 2924.7 - - Strong C-H, 2653.8 - - OH strong, 1712.4 - - C=O stretch, 1640.6 - - C=O Stretch (amide), 1460.6- - aromatic C=C

Fig. 25: FTIR spectrum of extracted IAA sample

a) EPS produced by AB3 b) Extracted EPS from AB3 c) Carbohydrate estimation of EPS



d) SEM analysis of EPS e) EDS analysis of EPS

Fig. 26: Extraction and compositional analysis of extracted EPS sample

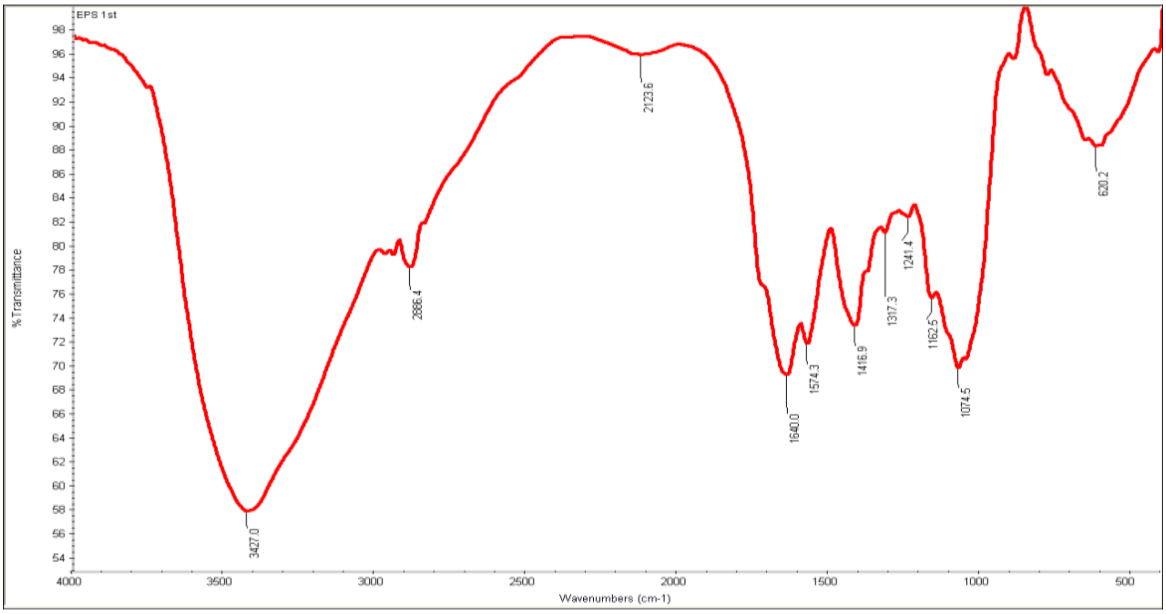


Fig. 27: FTIR spectrum of extracted EPS sample

(a) **(b)** **(c)**

Fig. 28: Root nodule formation in a) *Abrus, b) Sesbania* and c) *Leucaena*

(a) **(b)**

Fig. 29: Nodulation test for common legume crops: a) chickpea and b) mung)

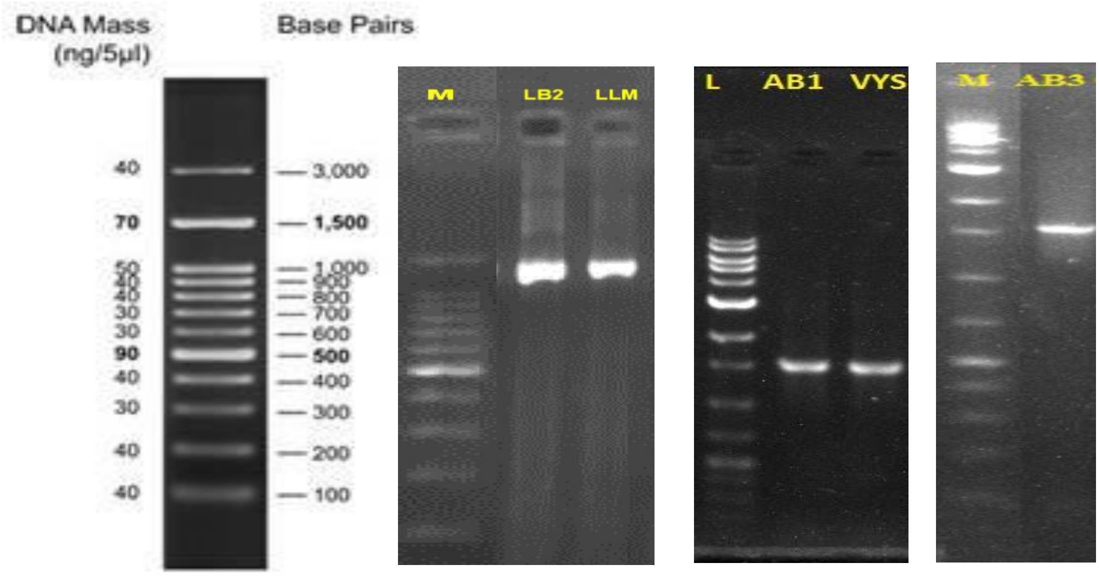


Fig. 30: Electrophoretic analysis of amplified product

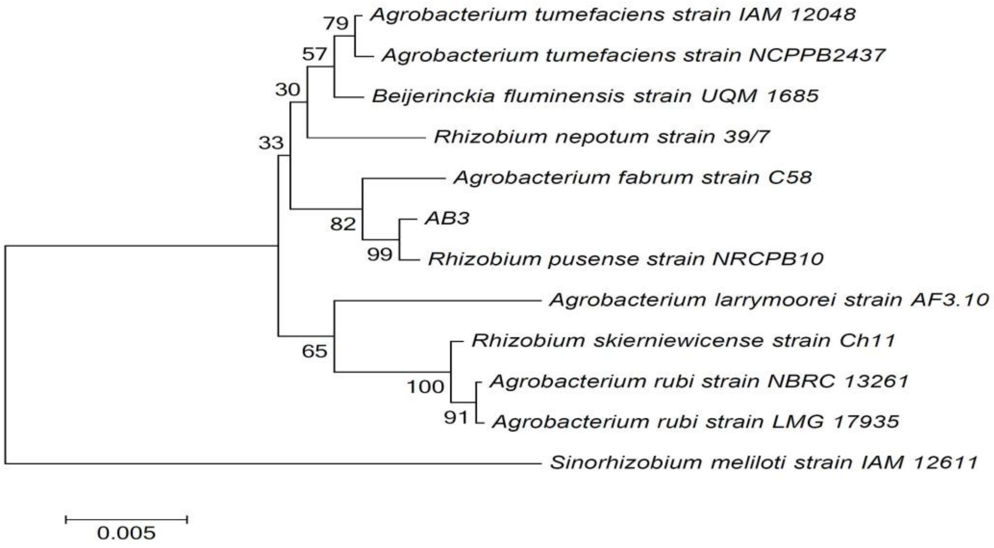


Fig. 31: Phylogenetic tree of isolate AB3

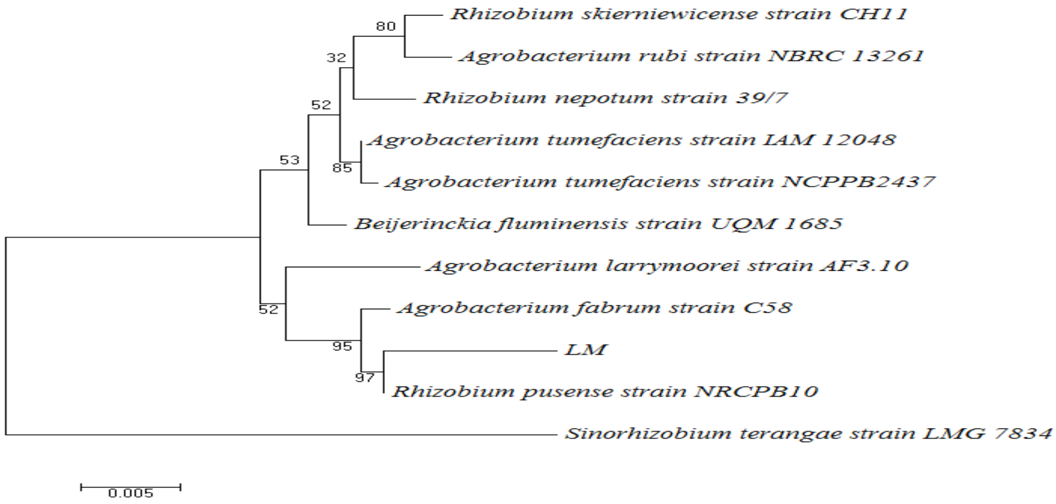


Fig. 32: Phylogenetic tree of isolate LM

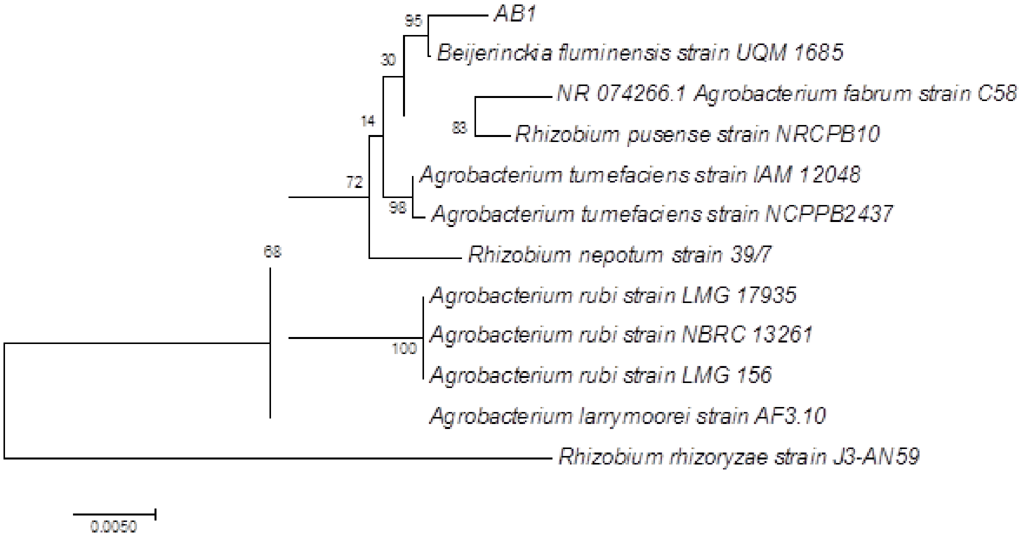


Fig. 33: Phylogenetic tree of isolate AB1

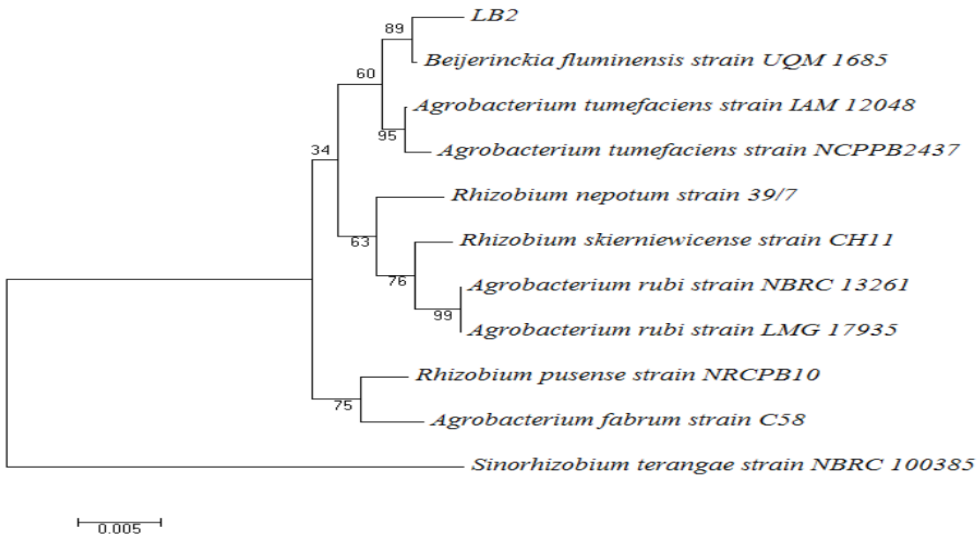


Fig. 34: Phylogenetic tree of isolate LB2

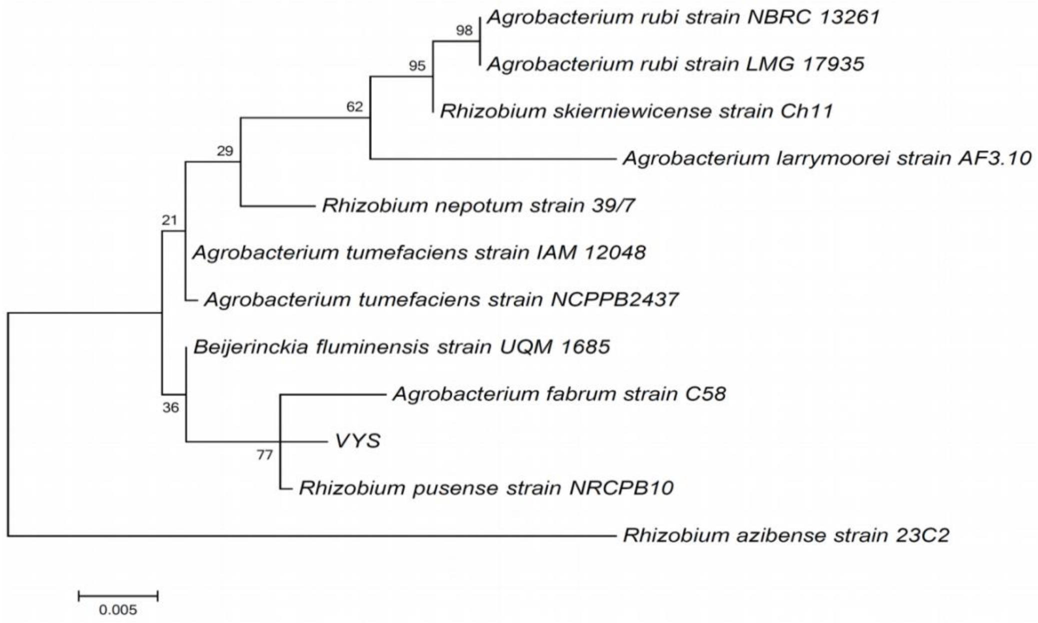


Fig. 35: Phylogenetic tree of isolate VYS

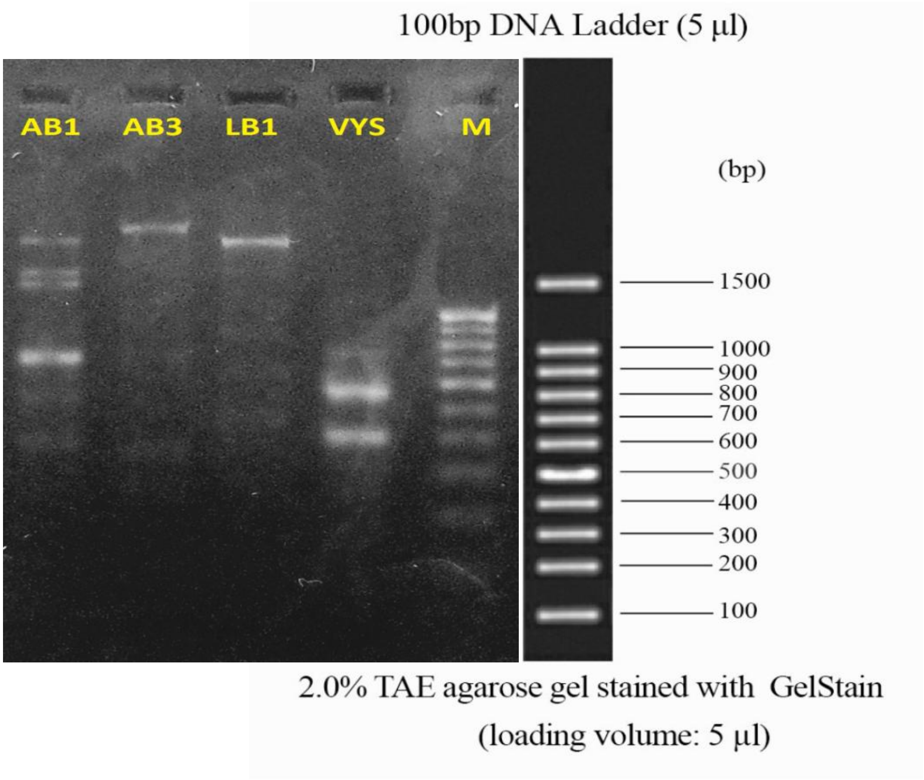


Fig. 36: Representative RAPD profiles of rhizobial isolates, amplification of the genomic DNA

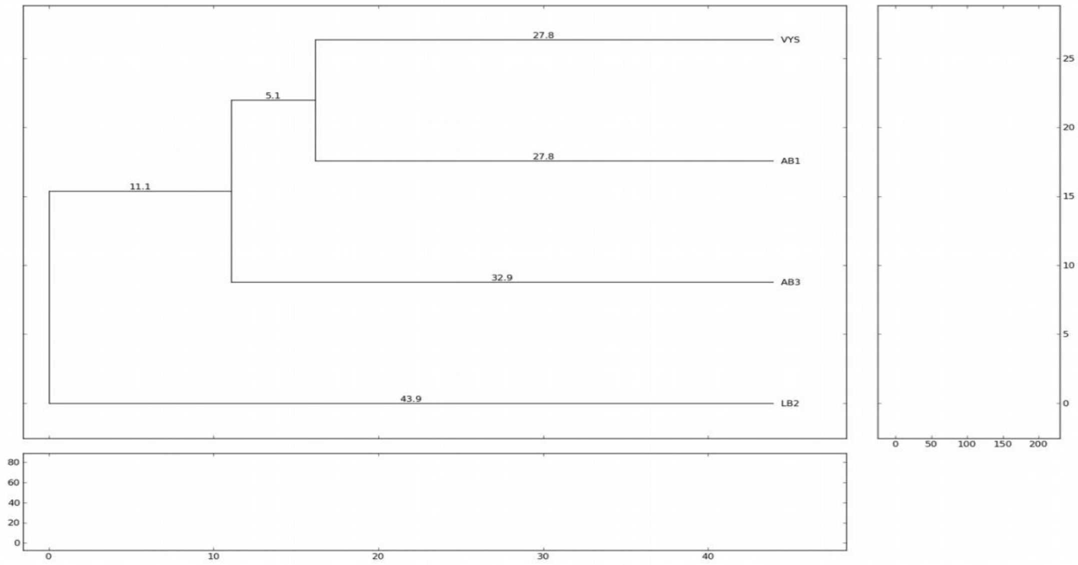


Fig. 37: Dendrogarm of rhizobial strains derived from RAPD fingerprints

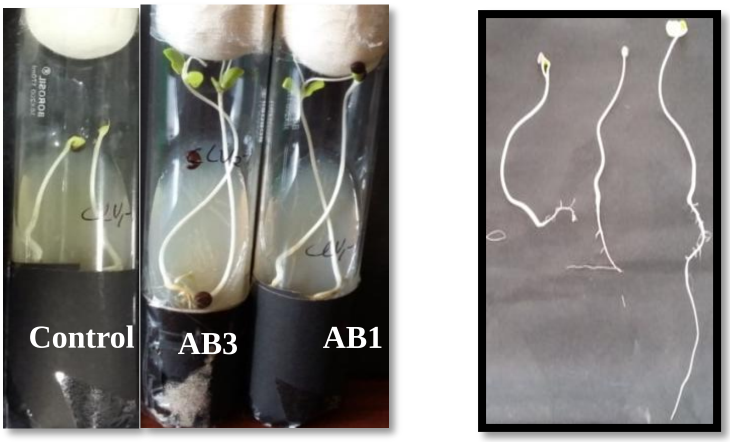
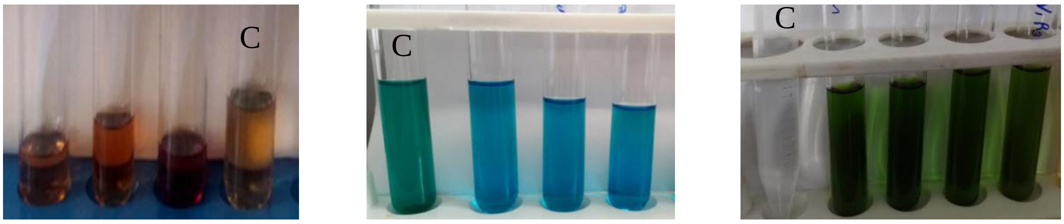
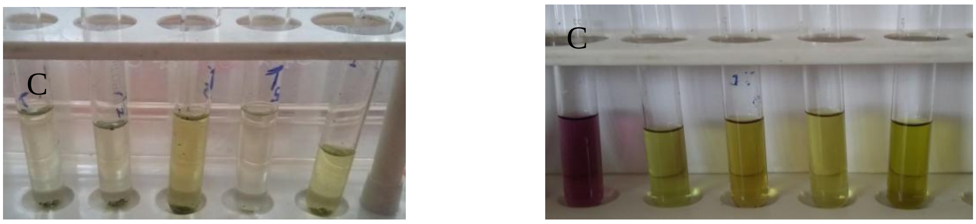


Fig. 38: *In vitro* applications of isolates on their host plant *Abrus*

Fig. 39: *In vivo* application of isolate on their host plant *Abrus*



Carbohydrate estimation Protein estimation Chlorophyll estimation



Estimation of flavonoid content Estimation of antioxidant activity

Fig. 40: Analysis of biochemical content of bacteria (AB1 and Ab3) treated plants

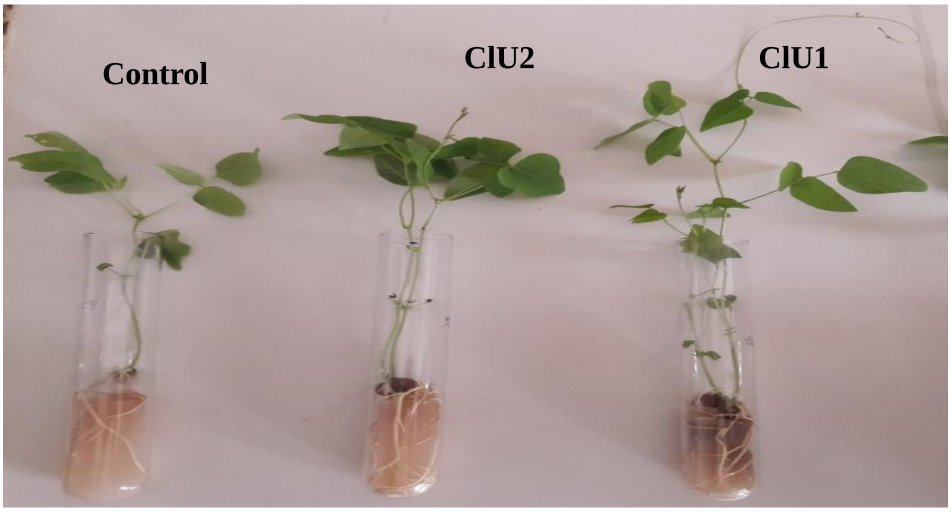


Fig. 41: *In vitro* applications of isolate on *Clitoria*

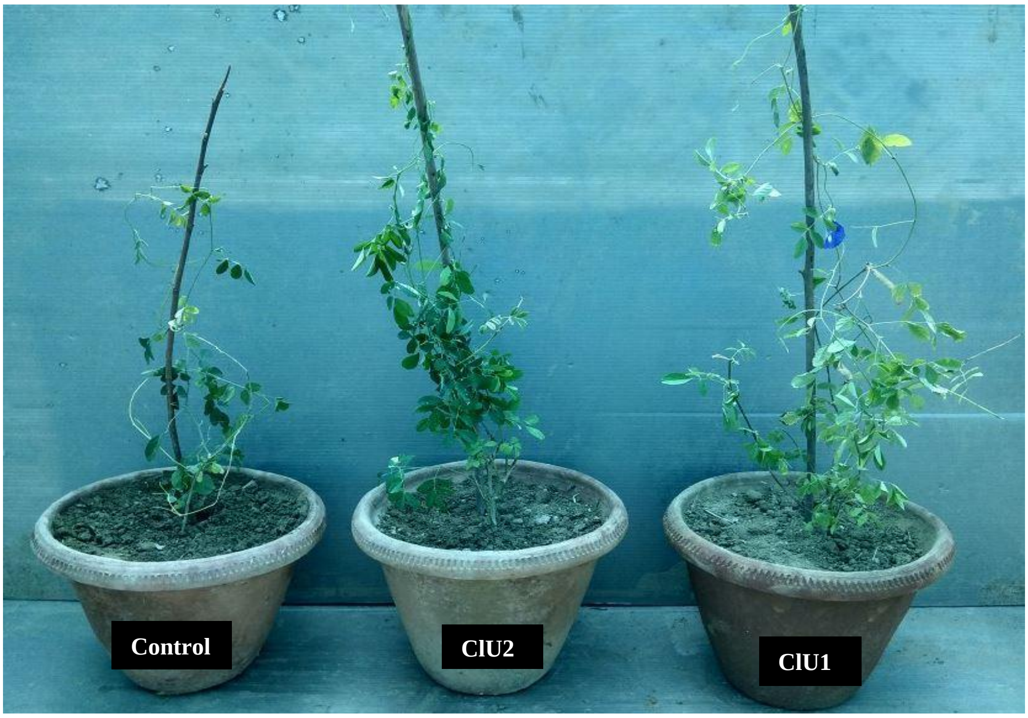


Fig. 42*:* *In vivo* application of isolate on *Clitoria*

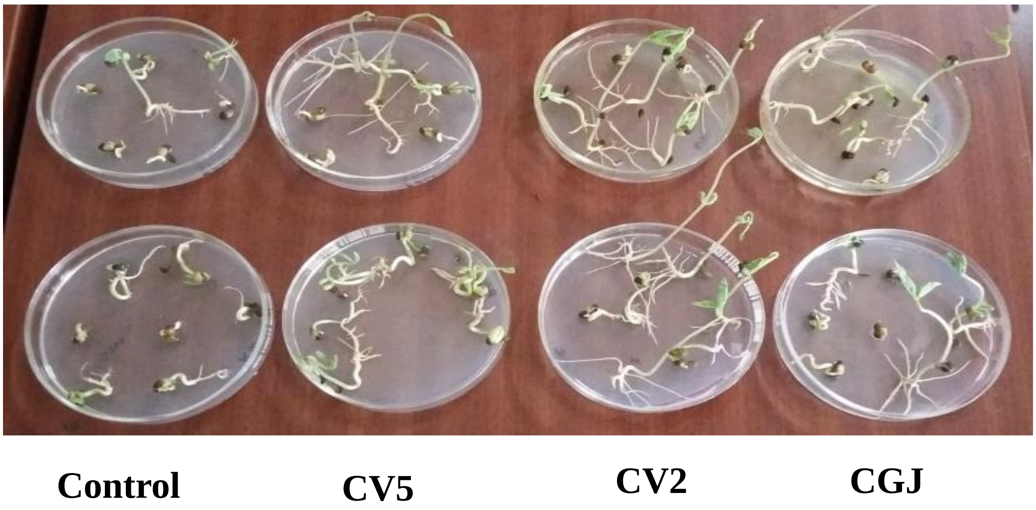


Fig. 43: *In vitro* applications of isolate on *Crotalaria*

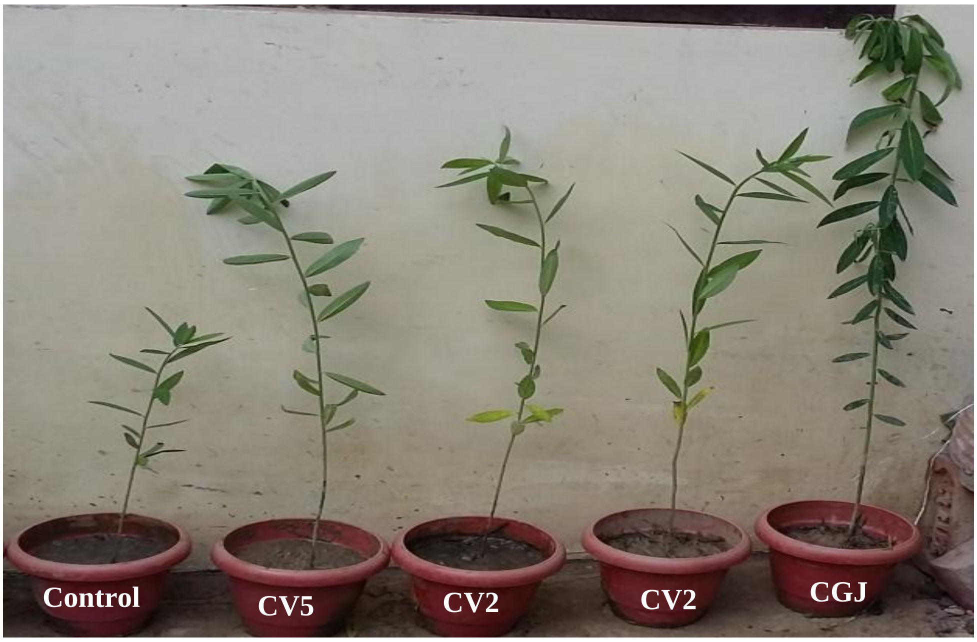


Fig. 44: *In vivo* applications of isolates on *Crotalaria*

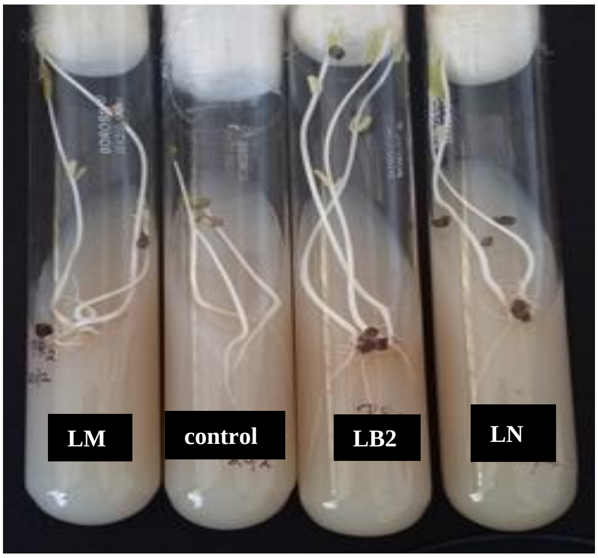
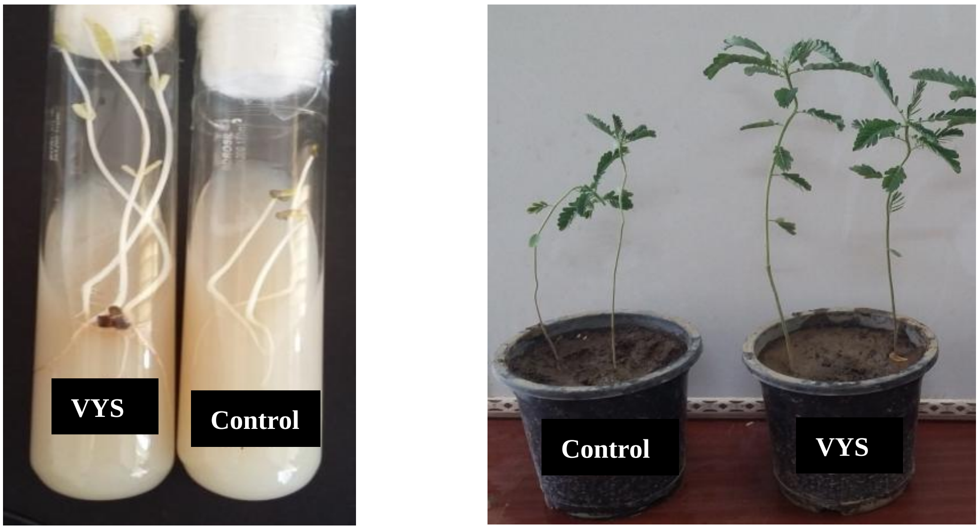


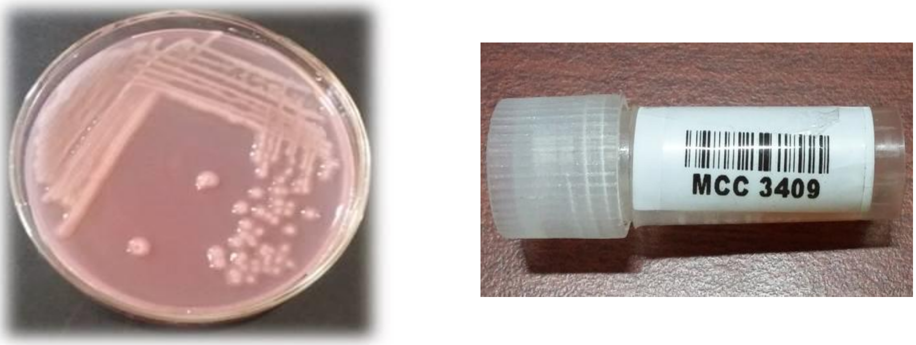
Fig. 45: *In vitro* applications of isolate on *Leucaena*

Fig. 46: *In vivo* applications of isolate on *Leucaena*



(a) **(b)**

Fig. 47: Application of isolate on *Sesbania* a) *in vitro*, b*) in vivo*



(Pure culture of AB3) (AB3 received from MCC)

Fig. 48: Type strain (*R. pusense* AB3) received from MCC, Pune after submission

Table 7: Detail of samples and sampling sites

S. No.	Plants name (Sample)	Collection sites	Nodule occupancy	Number of isolates	Name of isolates
1.	*Abrus prectorius*	Krishnanagar, Bijnaur	Medium with medium sized round shaped nodules	3	AB1, AB2, AB3
2.	*Clitoria ternatea*	BBAU campus, Rajajipurum	Medium with medium sized, round shaped nodules	4	ClU1, ClU2, ClR1, ClR2
3.	*Crotalaria juncea*	Vrindavan yojna, BBAU campus, Sarojini nagar, Banthara, Chinhat	High with large, medium and small sized, coralloid shaped nodules	14	CV1, CV2, CV3, CV4, CV5,CV6, CV7, CV8, CGJ, CBn, CSI, CB1, CB2, CB3
4.	*Leucaena leucocephala*	BBAU campus, Mohanlalganj, Nagram	Very less with small sized, round shaped nodules	4	LB1, LB2, LM , LN
5.	*Sesbania bispinosa*	Alamnagar, Amausi, Kakori, Kankaha, Khujauli, Madiyaon, Malihabad, Natwar, Kheda, Neelmattha, Para, Rahmankheda	Very high with large sized, round shaped nodules	15	HKS, PSS, UCS, VYS, SBn, SSI, SB1, SB2, SB3, SM, SN, SM1, SM2, KSI, KS2

Table 8: Phenotypic characters of isolates

S. No.	Isolates	Growth rate	Generation time (hrs)	Motility	Colony morphology		Microscopic observation	
					colour	appearance	Gram nature	shape
1.	AB1	Fast	2.9	+	white	mucilaginous	negative	rod
2.	AB2	Fast	3.0	+	white	mucilaginous	negative	rod
3.	AB3	Fast	2.9	+	white	mucilaginous	negative	rod
4.	ClU1	Fast	2.5	+	beige	mucilaginous	negative	rod
5.	ClU2	Fast	2.5	+	beige	mucilaginous	negative	rod
6.	ClR1	Fast	2.6	+	beige	mucilaginous	negative	rod
7.	ClR2	Fast	2.6	+	beige	mucilaginous	negative	rod
8.	CV1	Slow	7.1	+	white	mucilaginous	negative	rod
9.	CV2	Fast	3.5	+	beige	mucilaginous	negative	rod
10.	CV3	Fast	3.5	+	white	mucilaginous	negative	rod
11.	CV4	Fast	3.0	+	white	mucilaginous	negative	rod
12.	CV5	Fast	2.5	+	beige	mucilaginous	negative	rod
13.	CV6	Fast	2.5	+	beige	mucilaginous	negative	rod
14.	CV7	Slow	7.2	+	white	mucilaginous	negative	rod
15.	CV8	Slow	7.2	+	white	mucilaginous	negative	rod
16.	CGJ	Fast	3.5	+	white	mucilaginous	negative	rod
17.	CBn	Fast	3.5	+	beige	mucilaginous	negative	rod
18.	CSI	Fast	2.5	+	beige	mucilaginous	negative	rod
19.	CB1	Fast	3.0	+	white	mucilaginous	negative	rod
20.	CB2	Fast	3.0	+	white	mucilaginous	negative	rod
21.	CB3	Fast	3.0	+	white	mucilaginous	negative	rod
22.	LB1	Fast	3.5	+	white	mucilaginous	negative	rod
23.	LB2	Fast	3.0	+	white	mucilaginous	negative	rod
24.	LM	Fast	2.9	+	white	mucilaginous	negative	rod
25.	LN	Fast	2.5	+	beige	mucilaginous	negative	rod
26.	HKS	Fast	2.5	+	white	mucilaginous	negative	rod
27.	PSS	Fast	2.5	+	beige	mucilaginous	negative	rod

28.	UCS	Fast	2.6	+	beige	mucilaginous	negative	rod
29.	VYS	Fast	3.0	+	white	mucilaginous	negative	rod
30.	SBn	Fast	2.6	+	beige	mucilaginous	negative	rod
31.	SSI	Fast	2.6	+	beige	mucilaginous	negative	rod
32.	SB1	Slow	7.0	+	white	mucilaginous	negative	rod
33.	SB2	Slow	7.5	+	white	mucilaginous	negative	rod
34.	SB3	Slow	7.0	+	white	mucilaginous	negative	rod
35.	SM	Fast	2.5	+	beige	mucilaginous	negative	rod
36.	SN	Fast	2.5	+	beige	mucilaginous	negative	rod
37.	SM1	Fast	3.2	+	white	mucilaginous	negative	rod
38.	SM2	Fast	3.2	+	white	mucilaginous	negative	rod
39.	KS1	Fast	3.0	+	white	mucilaginous	negative	rod
40.	KS2	Fast	3.0	+	white	mucilaginous	negative	rod

+ = positive for test

Table 9: Carbon source utilization pattern of the isolates

	Carbon sources												
Isolates	**Mannitol**	**Glucose**	**Dextrose**	**Lactose**	**Galactose**	**Sucrose**	**Maltose**	**Starch**	**Glycerol**	**Fructose**	**Citrate**	**Malate**	**Trehalose**
AB1	+	+	+	+	+	+	+	-	-	-	-	+	-
AB2	+	+	+	+	+	+	+	+	-	-	+	+	-
AB3	+	+	+	+	+	+	+	+	-	-	-	+	-
ClU1	+	+	+	+	+	+	+	-	+	+	+	+	-
ClU2	+	+	+	+	+	+	+	-	+	+	+	+	-
ClR1	+	+	+	+	+	+	+	-	+	+	+	+	-
ClR2	+	+	+	+	+	+	+	-	+	+	+	+	-
CV1	+	+	+	+	+	+	+	-	-	+	+	+	-
CV2	+	+	+	+	+	+	+	-	-	+	+	+	-
CV3	+	+	+	+	+	+	+	+	-	+	+	+	-
CV4	+	+	+	+	+	+	+	+	+	+	+	+	-
CV5	+	+	+	+	+	+	+	+	+	-	+	+	-
CV6	+	+	+	+	+	+	+	+	+	-	+	+	-
CV7	+	+	+	+	+	+	+	+	+	-	+	+	-
CV8	+	+	+	+	+	+	+	+	+	-	+	+	-
CGJ	+	+	+	+	+	+	+	+	+	+	+	+	-
CBn	+	+	+	+	+	+	+	+	+	+	+	+	-
CSI	+	+	+	+	+	+	+	+	+	+	+	+	+
CB1	+	+	+	+	+	+	+	+	-	+	+	+	+
CB2	+	+	+	+	+	+	+	+	-	+	+	+	+
CB3	+	+	+	+	+	+	+	+	-	+	+	+	+
LB1	+	+	+	+	+	+	+	+	+	-	-	+	-
LB2	+	+	+	+	+	+	+	+	+	-	+	+	-
LM	+	+	+	+	+	+	+	+	+	-	-	+	-

LN	+	+	+	+	+	+	+	+	+	-	+	+	+
HKS	+	+	+	+	+	+	+	+	+	+	+	-	+
PSS	+	+	+	+	+	+	+	+	+	+	+	-	-
UCS	+	+	+	+	+	+	+	+	+	+	+	+	-
VYS	+	+	+	+	+	+	+	+	+	+	-	+	-
SBn	+	+	+	+	+	+	+	+	+	+	+	+	-
SSI	+	+	+	+	+	+	+	+	+	+	+	+	-
SB1	+	+	+	+	+	+	+	+	+	+	-	+	-
SB2	+	+	+	+	+	+	+	-	+	+	+	+	-
SB3	+	+	+	+	+	+	+	-	+	+	-	+	-
SM	+	+	+	+	+	+	+	-	-	-	+	+	+
SN	+	+	+	+	+	+	+	-	-	-	+	+	+
SM1	+	+	+	+	+	+	+	+	-	-	-	-	+
SM2	+	+	+	+	+	+	+	-	-	-	+	-	+
KS1	+	+	+	+	+	+	+	+	+	+	+	+	+
KS2	+	+	+	+	+	+	+	-	+	+	+	+	+

+ =utilized, - = not utilized

Table 10: Nitrogen source utilization pattern of the isolates

Isolates	Nitrogen sources										
	Yeast extract	KNO_3	$NaNO_3$	NH_4Cl	NH_4So_4	Glutamine	Tryptophan	Glycine	Cysteine	Lysine	Methionine
AB1	+	+	+	+	+	+	+	-	+	+	-
AB2	+	+	+	+	+	+	+	-	+	+	-
AB3	+	+	+	+	+	+	+	-	+	+	-
ClU1	+	+	+	+	+	+	+	-	+	+	-
ClU2	+	+	+	+	+	+	+	-	+	+	-
ClR1	+	+	+	+	+	+	+	-	+	+	-
ClR2	+	+	+	+	+	+	+	-	+	+	-
CV1	+	+	+	+	+	+	+	-	-	+	+
CV2	+	+	+	+	+	+	+	-	-	+	+
CV3	+	+	+	+	+	+	+	-	-	+	+
CV4	+	+	+	+	+	+	+	-	+	+	-
CV5	+	+	+	+	+	+	+	-	+	+	-
CV6	+	+	+	+	+	+	+	-	+	+	-
CV7	+	+	+	+	+	+	+	-	-	+	-
CV8	+	+	+	+	+	+	+	-	-	+	-
CGJ	+	+	+	+	+	+	+	-	+	+	-
CBn	+	+	+	+	+	+	+	-	+	+	-
CSI	+	+	+	+	+	+	+	-	+	+	-
CB1	+	+	+	+	+	+	+	-	+	-	-
CB2	+	+	+	+	+	+	+	-	+	+	-
CB3	+	+	+	+	+	+	+	-	+	-	-
LB1	+	+	+	+	+	+	+	-	+	+	-
LB2	+	+	+	+	+	+	+	-	+	+	-
LM	+	+	+	+	+	+	+	-	+	+	-

LN	+	+	+	+	+	+	+	-	+	+	-
HKS	+	+	+	+	+	+	+	-	+	+	+
PSS	+	+	+	+	+	+	+	-	+	+	+
UCS	+	+	+	+	+	+	+	-	+	+	+
VYS	+	+	+	+	+	+	+	-	+	+	-
SBn	+	+	+	+	+	+	+	-	-	+	-
SSI	+	+	+	+	+	+	+	-	-	+	-
SB1	+	+	+	+	+	+	+	-	-	+	-
SB2	+	+	+	+	+	+	+	-	-	-	-
SB3	+	+	+	+	+	+	+	-	-	+	-
SM	+	+	+	+	+	+	+	-	-	+	-
SN	+	+	+	+	+	+	+	-	-	+	-
SM1	+	+	+	+	+	+	+	-	-	-	-
SM2	+	+	+	+	+	+	+	-	-	-	-
KS1	+	+	+	+	+	+	+	-	-	+	-
KS2	+	+	+	+	+	+	+	-	-	+	-

+ = utilized, - = not utilized

Table 11: Growth pattern of isolates under different salt concentrations, temperatures and pH

Isolates	Salt concentration (%)					pH variants					temperature				
	5	**6**	**7**	**8**	**9**	**4**	**6**	**8**	**10**	**12**	**5**	**15**	**25**	**35**	**45**
AB1	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-
AB2	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-
AB3	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-
ClU1	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-
ClU2	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
ClR1	+	+	+	-	-	-	+	+	+	+	-	+	+	+	-
ClR2	+	+	+	+	-	-	+	+	+	+	-	+	+	+	-
CV1	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
CV2	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-
CV3	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
CV4	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
CV5	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-
CV6	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-
CV7	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
CV8	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
CGJ	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-
CBn	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-
CSI	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-
CB1	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
CB2	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
CB3	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
LB1	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
LB2	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-
LM	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-

LN	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-
HKS	+	+	-	-	-	+	+	+	+	+	-	+	+	+	-
PSS	+	+	-	-	-	+	+	+	+	+	-	+	+	+	-
UCS	+	+	-	-	-	+	+	+	+	+	-	+	+	+	-
VYS	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
SBn	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
SSI	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-
SB1	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
SB2	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
SB3	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-
SM	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-
SN	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-
SM1	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-
SM2	+	+	+	+	-	-	+	+	+	+	-	+	+	+	-
KS1	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-
KS2	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-

+ = positive for test, - = negative for test

Table 12: Intrinsic antibiotic activity test of isolates:

Isolates	Antibiotics (sensitivity index in cm)									
	Tetracycline	Chloramphenicol	Penicillin	Ampicillin	Neomycin	Gentamicin	Kanamycin	Erythromycin	Nalidixic acid	Streptomycin
AB1	3.2	3.0	R	1.1	1.4	1.8	2.4	1.6	3.0	1.7
AB2	3.1	3.0	R	1.2	1.3	1.6	2.3	1.5	3.1	1.6
AB3	3.4	2.0	R	3.1	1.7	1.7	2.2	2.3	2.4	2.5
ClU1	2.9	2.6	R	1.5	1.6	1.5	1.7	1.5	2.3	2.1
ClU2	2.1	2.4	R	1.5	1.9	1.9	1.6	1.4	2.4	1.6
ClR1	2.2	2.1	R	1.3	1.8	1.8	1.7	1.5	2.3	2.1
ClR2	2.2	2.2	R	1.2	1.6	1.8	1.7	1.5	2.1	2.0
CV1	2.5	2.4	R	1.2	1.8	1.8	2.0	1.4	2.5	2.8
CV2	2.8	3.0	R	1.1	2.0	2.3	2.2	1.4	2.5	2.8
CV3	2.5	2.9	R	1.3	1.6	2.1	1.6	1.3	2.6	2.7
CV4	2.4	2.8	R	1.1	1.5	2.1	1.8	1.6	2.4	2.6
CV5	2.5	2.7	R	2.1	1.4	2.1	1.8	1.8	2.5	2.7
CV6	3.0	2.8	R	1.5	1.5	1.6	1.6	1.6	2.1	2.7
CV7	2.8	2.6	R	1.2	1.3	1.9	1.5	1.2	2.3	2.5
CV8	2.3	2.7	R	1.2	1.2	2.1	1.5	1.5	2.3	2.5
CGJ	2.5	2.8	R	1.2	1.3	2.0	2.4	1.8	2.1	2.8
CBn	2.6	2.5	R	1.1	1.4	1.9	2.2	2.1	3.0	2.5
CSI	2.6	2.6	R	1.4	1.5	1.8	2.1	2.0	2.8	2.2
CB1	2.7	2.5	R	1.1	1.5	1.8	2.0	2.1	2.8	2.1
CB2	1.9	2.5	R	1.0	1.7	1.6	2.1	R	2.3	2.5
CB3	2.2	2.7	R	1.1	1.8	2.1	2.2	0.9	2.0	2.1
LB1	2.3	2.5	R	1.2	1.9	1.6	1.8	0.9	2.2	2.0
LB2	3.2	2.1	R	1.1	2.3	2.2	1.3	1.8	2.1	2.1

LM	3.3	3.3	R	1.9	2.1	2.5	1.8	2.3	2.0	2.2
LN	2.4	2.6	R	1.5	2.0	1.9	2.2	1.1	2.2	2.0
HKS	2.7	2.5	R	1.7	1.9	1.8	2.1	1.6	2.8	2.5
PSS	2.4	2.5	R	1.5	1.8	1.9	2.1	1.7	2.7	1.8
UCS	2.2	2.4	R	1.2	1.8	1.9	1.9	1.8	2.8	1.7
VYS	3.5	1.7	R	2.5	2.4	2.2	1.5	2.6	2.3	1.7
SBn	2.9	1.9	R	2.3	2.1	1.8	1.8	1.8	2.6	1.6
SSI	1.9	1.9	R	2.1	2.0	1.5	1.7	1.8	2.6	1.6
SB1	2.8	2.5	R	1.6	1.4	1.1	1.8	R	2.0	1.5
SB2	2.5	2.5	R	R	1.7	2.0	2.3	1.1	2.4	1.6
SB3	2.1	2.2	R	1.8	1.9	1.7	2.1	1.5	2.5	1.5
SM	3.1	2.7	R	1.1	1.8	1.8	2.1	1.2	2.0	1.7
SN	2.4	2.3	R	1.2	1.9	1.7	2.0	1.4	2.2	1.8
SM1	3.3	3.0	R	1.2	2.0	1.5	2.4	1.7	2.1	1.8
SM2	2.5	2.2	R	1.1	1.7	1.4	2.3	1.6	2.5	1.6
KS1	3.0	3.0	R	R	2.1	2.0	1.9	1.2	2.2	1.8
KS2	2.5	2.8	R	1.1	1.8	2.1	1.8	1.3	2.1	1.6

R= resistant for antibiotic

Table 13: Biochemical characters of isolates

Isolates	Biochemical test																		
	Catalase	**Oxidase**	**Lactose test**	**Growth on GPA**	**Growth on HAM**	**Growth in 8% KNO_3**	**Nitrate reduction**	**Urease**	**Gelatinase**	**Citrate utilization**	**Amylase**	**Lipase**	**Protease**	**Cellulase**	**Ammonia production**	**PHB production**	**Indole**	**Methyl Red**	**Voges Proskauer**
AB1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	+
AB2	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	+
AB3	+	+	-	+	-	+	+	+	-	-	+	-	+	-	+	+	+	+	-
ClU1	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	+	+	-
ClU2	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	++	-	-	+
ClR1	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	+
ClR2	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	+
CV1	+	+	-	+	-	+	+	-	-	+	-	-	+	-	+	++	+	+	-
CV2	+	+	-	+	-	+	+	-	-	+	-	-	+	-	+	+	+	+	-
CV3	+	+	-	+	-	-	+	-	-	+	+	-	+	-	+	++	+	-	+
CV4	+	+	-	+	-	-	+	-	-	+	+	-	+	-	+	-	-	-	+
CV5	+	+	-	-	-	+	+	-	-	+	+	-	+	-	+	+	+	+	-
CV6	+	+	-	-	-	+	+	-	-	+	+	-	-	-	+	++	+	+	-
CV7	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	++	-	+	-
CV8	+	+	-	+	-	+	+	-	-	+	+	-	-	-	+	-	-	+	-
CGJ	+	+	-	-	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-
CBn	+	+	-	+	-	+	+	-	-	+	+	-	+	-	+	+	-	+	-
CSI	+	+	-	+	-	+	+	+	-	+	+	-	+	-	+	+	-	+	-
CB1	+	+	-	+	-	+	+	+	-	+	+	-	-	-	+	+	-	+	-
CB2	+	+	-	+	-	+	+	+	-	+	+	-	-	-	+	+	-	-	+
CB3	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+	-
LB1	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	-	+

LB2	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	+
LM	+	+	-	+	-	+	+	+	-	-	+	-	+	-	+	+	+	-	+
LN	+	+	+	-	+	+	+	-	-	+	+	-	-	-	+	+	+	+	-
HKS	+	+	-	-	-	-	+	+	-	+	+	-	-	-	+	+	-	+	-
PSS	+	+	-	-	-	-	+	-	-	+	+	-	-	-	+	-	-	+	-
UCS	+	+	-	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	-
VYS	+	+	-	+	-	+	+	+	-	-	+	-	-	-	+	+	+	+	-
SBn	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	-	-	+	-
SSI	+	+	-	+	-	+	+	-	-	+	+	-	+	-	+	+	-	+	-
SB1	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	++	+	-	+
SB2	+	+	-	+	-	+	+	-	-	+	-	-	-	-	+	++	-	+	-
SB3	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	+	-	+
SM	+	+	-	+	-	+	+	-	-	+	-	-	-	-	+	+	-	+	-
SN	+	+	+	+	+	+	+	-	-	+	-	+	-	-	+	-	-	-	+
SM1	+	+	+	-	+	+	+	+	-	-	+	-	+	-	+	+	-	-	+
SM2	+	+	-	-	-	+	+	+	-	+	-	-	+	-	+	+	-	+	-
KS1	+	+	-	-	-	-	+	+	-	+	+	-	-	-	+	+	-	+	-
KS2	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	-	+	-

+ = positive for test, ++ = more positive for test, - = negative for test

Table 14: Plant growth promoting properties of isolates (quantitative and qualitative)

Isolates	**PGP characters**									
	Nitrogen fixation	**Phosphate solubilisation (psi)**	**Zinc solubilisation (zsi)**	**IAA production (µg/ml)**		**Gibberellin production (µg/ml)**	**EPS production (µg/ml)**	**Siderophore production (psu)**		**HCN production**
				Traditional method	**Microplate method**			**Traditional method**	**Microplate method**	
AB1	+	**+ (1.8)**	**++ (2.1)**	**+ (89.78)**	**+ (90.01)**	**+ (1.2)**	**+ (50)**	**+ (07.97)**	**+ (08.33)**	**-**
AB2	+	+ (1.5)	+ (0.9)	+ (85.12)	+ (85.34)	++ (2.4)	+ (52)	-	-	-
AB3	**++**	**+ (2.3)**	**+ (2.5)**	**++ (120.1)**	**++ (120.0)**	**+ (1.1)**	**++ (75)**	**+ (33.34)**	**+ (33.61)**	**-**
ClU1	**++**	**+ (1.8)**	**+ (1.2)**	**+ (75.67)**	**+ (75.87)**	**+++ (3.0)**	**+ (60)**	**+ (30.39)**	**+ (30.64)**	**-**
ClU2	**+**	**+ (1.9)**	**+ (1.8)**	**+ (70.12)**	**+ (70.56)**	**++ (2.5)**	**+ (61)**	**+ (21.46)**	**+ (21.90)**	**-**
ClR1	+	+ (0.8)	-	-	-	-	+ (50)	-	-	-
ClR2	+	+ (0.9)	-	-	-	+ (1.3)	+ (51)	-	-	-
CV1	+	-	+ (1.8)	-	-	-	+ (40)	-	-	-
CV2	**+**	**+ (1.0)**	**+ (2.0)**	**+ (90.12)**	**+ (90.54)**	**+ (1.2)**	**+ (45)**	**+ (27.82)**	**+ (27.90)**	**-**
CV3	+	-	+ (0.9)	-	-	-	+ (42)	-	-	-
CV4	+	+ (0.8)	+ (1.5)	-	-	-	+ (40)	-	-	-
CV5	**+**	**+ (2.0)**	**+ (2.3)**	**+ (70.34)**	**+ (70.76)**	**++ (2.6)**	**+ (67)**	**+ (35.51)**	**+ (35.77)**	**-**
CV6	+	+ (1.8)	+ (1.9)	+ (72.31)	+ (72.76)	+ (1.6)	+ (68)	+ (25.12)	+ (25.31)	-
CV7	+	-	+ (1.8)	-	-	+ (1.5)	+ (63)	-	-	-
CV8	+	-	+ (2.0)	-	-	+ (1.6)	+ (60)	-	-	-
CGJ	**+**	**+ (1.2)**	**+ (2.3)**	**+ (80.12)**	**+ (80.76)**	**-**	**+ (64)**	**+ (18.97)**	**+ (19.01)**	**-**
CBn	+	+ (1.1)	+ (2.0)	+ (69.13)	+ (69.78)	+ (1.8)	+ (45)	+ (21.12)	+ (21.32)	-
CSI	+	+ (1.0)	+ (2.1)	-	-	+ (1.9)	+ (43)	+ (25.12)	+ (25.67)	-
CB1	+	+ (0.9)	+ (1.5)	+ (85.78)	+ (85.90)	+ (1.7)	+ (42)	-	-	-
CB2	+	-	+ (0.9)	-	-	-	+ (42)	+ (12.37)	+ (12.46)	-
CB3	+	-	+ (2.0)	-	-	-	+ (46)	-	-	-

LB1	+	+ (1.5)	+ (0.8)	+ (85.45)	+ (85.67)	-	+ (68)	+ (30.12)	+ (30.09)	-
LB2	**++**	**+ (2.2)**	**++ (2.6)**	**++ (110.7)**	**++ (110.9)**	**+ (1.6)**	**++ (70)**	**+ (45.12)**	**+ (45.64)**	**-**
LM	**++**	**+ (2.1)**	**++ (2.4)**	**+ (90.67)**	**+ (90.89)**	**+ (1.5)**	**+ (60)**	**+ (12.63)**	**+ (13.01)**	**-**
LN	**+**	**+ (2.2)**	**+ (1.2)**	**+ (78.34)**	**+ (78.59)**	**++ (2.7)**	**+ (69)**	**+ (40.44)**	**+ (41.44)**	**-**
HKS	+	+ (1.5)	-	-	-	-	+ (70)	-	-	-
PSS	+	+ (1.6)	-	-	-	-	+ (65)	-	-	-
UCS	+	+ (1.2)	-	+ (65.12)	+ (65.98)	-	+ (66)	-	-	-
VYS	**++**	**+ (2.0)**	**+ (1.9)**	**+ (80.56)**	**+ (80.79)**	**+ (1.3)**	**+ (62)**	**+ (27.31)**	**+ (27.45)**	**-**
SBn	+	+ (1.2)	-	+ (76.98)	+ (77.01)	+ (1.2)	+ (56)	-	-	-
SSI	+	+ (1.3)	-	-	-	+ (1.1)	+ (67)	+	-	-
SB1	+	+ (1.2)	+ (1.5)	+ (65.45)	+ (65.67)	+ (0.9)	+ (53)	-	-	-
SB2	+	+ (1.0)	+ (1.4)	+ (69.12)	+ (69.43)	+ (1.4)	+ (54)	+ (24.15)	+ (24.45)	-
SB3	+	-	-	+ (60.65)	+ (60.89)	-	+ (52)	-	-	-
SM	+	+ (0.9)	+ (0.6)	+ (55.76)	+ (55.90)	+ (1.5)	+ (58)	+ (15.12)	+ (15.52)	-
SN	-	+ (0.7)	+ (1.8)	-	-	+ (1.6)	+ (45)	+ (23.21)	+ (23.32)	-
SM1	+	-	-	+ (55.73)	+ (55.89)	-	+ (55)	-	-	+
SM2	+	+ (1.4)	-	+ (54.21)	+ (54.56)	-	+ (56)	-	-	-
KS1	+	+ (1.2)	-	-	-	-	+ (58)	-	-	-
KS2	+	+ (1.2)	-	-	-	-	+ (59)	-	-	-

+ = positive for test, ++ = more positive for test, - = negative for test, psi= phosphate solubilisation index, zsi= zinc solubilisation index, psu= percent siderophore unit

Table 15: Biocontrol activity of isolates against common phytopathogens

Isolates	Phytopathogens (% radial growth inhibition)		
	F. moniliforme	*F. oxysporum*	*F. solani*
AB1	-	-	-
AB3	++ (60.12)	+ (39.13)	+ (40.12)
ClU1	+ (49.54)	-	-
ClU2	+ (50.32)	-	+ (32.45)
ClR1	-	-	-
ClR2	-	-	-
CV1	-	-	-
CV2	+ (42.15)	+ (43.64)	-
CV3	-	-	-
CV4	-	-	-
CV5	+ (48.23)	+ (44.12)	+ (42.12)
CV6	-	-	-
CV7	-	-	-
CV8	-	-	-
CGJ	-	-	-
CBn	-	-	-
CSI	-	-	-
CB1	-	-	-
CB2	-	-	-
CB3	-	-	-
LB1	-	-	-
LB2	++ (58.32)	+ (48.43)	+ (55.34)
LM	+ (49.12)	-	+ (39.12)
LN	+ (53.54)	-	-
HKS	+ (47.31)	-	-
PSS	+ (35.21)	-	-
UCS	+ (37.65)	-	-
VYS	+ (43.54)	-	+ (41.54)
SBn	-	-	-
SSI	-	-	-
SB1	-	-	-
SB2	-	-	-
SB3	-	-	-
SM	-	-	-
SN	-	-	-
SM1	+ (37.87)	-	-
SM2	+ (35.87)	-	-
KS1	-	-	-
KS2	-	-	-

Table 16: Nodulation test of isolates on their host plant and common legume crops

S. No.	Isolates	Wild legumes					Legume crops				
		Abrus	***Clitoria***	***Crotalaria***	***Leucaena***	***Sesbania***	**Cowpea**	**Chickpea**	**Mung**	**Urd**	**Methi**
1.	AB1	-	-	-	-	-	-	-	-	-	-
2.	AB3	+	-	-	-	-	-	-	-	-	-
3.	ClU1	-	-	-	-	-	-	-	-	-	-
4.	ClU2	-	-	-	-	-	-	-	-	-	-
5.	CV2	-	-	-	-	-	-	-	-	-	-
6.	CV5	-	-	-	-	-	-	-	-	-	-
7.	CGJ	-	-	-	-	-	-	-	-	-	-
8.	LM	-	-	-	+	-	-	-	-	-	-
9.	LB2	-	-	-	-	-	-	-	-	-	-
10.	LN	-	-	-	-	-	-	-	-	-	-
11.	VYS	-	-	-	-	+	-	-	-	-	-

+ = positive for nodulation, - = negative for nodulation

Table 17: Genotypic characters and NCBI-Genbank submission detail of isolates

S. No.	Isolates	Host plant	Identified as	Accession No.
1.	AB1	*Abrus*	*Beijerinckia fluminensis*	MF400858
2.	AB3	*Abrus*	*Rhizobium pusense*	KY392993
3.	ClU1	*Clitoria*	*Kosakonia sacchari*	KY392994
4.	ClU2	*Clitoria*	*Enterobacter cloacae*	KY178303
5.	CV2	*Crotalaria*	*Pantoea agglomerans*	KY178304
6.	CV5	*Crotalaria*	*Enterobacter cloacae*	MF416432
7.	CGJ	*Crotalaria*	*Cronobacter sakazakii*	MF416433
8.	LM	*Leucaena*	*Rhizobium pusense*	KY392995
9.	LB2	*Leucaena*	*Rhizobium radiobacter*	KY392996
10.	LN	*Leucaena*	*Kosakonia pseudosacchari*	KY392997
11.	VYS	*Sesbania*	*Rhizobium pusense*	-

Table 18: Diversity indices among all the isolates in respect to their host plant

5 Plants					
No	**Plants**	**Value**	**x**	**x^2**	**-x ln(x)**
1	*Abrus*	3	7.5%	0.006	0.194
2	*Clitoria*	4	10.0%	0.010	0.230
3	*Crotalaria*	14	35.0%	0.122	0.367
4	*Leucaena*	4	10.0%	0.010	0.230
5	*Sesbania*	15	37.5%	0.141	0.368
R1	**Simpson Dominance**			0.2888	
R2	**Shannon Entropy**				1.3900

Diversity Indices	
Index	**Value**
Number of Classes *N*	5
Richness *R*	5
Berger Parker Index p_{imax}	37.5%
Shannon Entropy$^{1)}$ *H* (nat)	1.3900
Shannon Entropy$^{1)}$ *H* (bit)	2.0054
Number Eq. ^1D (True Diversity)	4.0
Shannon Equitability *H*/ln*N*	86.4%
Simpson Dominance *SD*	28.9%
SD (unbiased - finite samples)	27.1%
True Diversity ^2D (Order 2)	3.5
Gini-Simpson Index 1-*SD*	71.1%
Gini-Simpson Equitability	88.9%
$^{1)}$**sometimes referred to as Shannon-Weaver or Shannon-Wiener Index**	

Table 19: Diversity indices among all the genetically identified rhizobial and non-rhiozbial isolates

Six bacterial genera					
No	**Bacteria**	**Value**	**x**	**x^2**	**-x ln(x)**
1	*Rhizobium*	4	36.4%	0.132	0.368
2	*Enterobacter*	2	18.2%	0.033	0.310
3	*Kosakonia*	2	18.2%	0.033	0.310
4	*Pantoea*	1	9.1%	0.008	0.218
5	*Beijerinckia*	1	9.1%	0.008	0.218
6	*Chronobacter*	1	9.1%	0.008	0.218
R1	**Simpson Dominance**			0.2231	
R2	**Shannon Entropy**				1.6417

Diversity Indices	
Index	**Value**
Number of Classes *N*	6
Richness *R*	6
Berger Parker Index p_{imax}	36.4%
Shannon Entropy$^{1)}$ *H* (nat)	1.6417
Shannon Entropy$^{1)}$ *H* (bit)	2.3685
Number Eq. ^{1}D (True Diversity)	5.2
Shannon Equitability *H*/ln*N*	91.6%
Simpson Dominance *SD*	22.3%
SD (unbiased - finite samples)	14.5%
True Diversity ^{2}D (Order 2)	4.5
Gini-Simpson Index 1-*SD*	77.7%
Gini-Simpson Equitability	93.2%

[1)]**sometimes referred to as Shannon-Weaver or Shannon-Wiener Index**

Table 20: Diversity indices among rhizobial isolates

Three bacterial genera					
No	**Bacteria**	**Value**	**x**	**x^2**	**-x ln(x)**
1	*R. pusense*	3	60.0%	0.360	0.306
2	*R. radiobacter*	1	20.0%	0.040	0.322
3	*B. flumensis*	1	20.0%	0.040	0.322
R1	**Simpson Dominance**			0.4400	
R2	**Shannon Entropy**				0.9503

Diversity Indices	
Index	**Value**
Number of Classes N	3
Richness R	3
Berger Parker Index p_{imax}	60.0%
Shannon Entropy[1)] H (nat)	0.9503
Shannon Entropy[1)] H (bit)	1.3710
Number Eq. ^1D (True Diversity)	2.6
Shannon Equitability $H/\ln N$	59.0%
Simpson Dominance SD	44.0%
SD (unbiased - finite samples)	30.0%
True Diversity ^2D (Order 2)	2.3
Gini-Simpson Index 1-SD	56.0%
Gini-Simpson Equitability	70.0%
[1)]**sometimes referred to as Shannon-Weaver or Shannon-Wiener Index**	

Table 21*: In-vitro* study of isolates on growth of *Abrus* plant

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)	Chlorophyll (mg/L)
Control	77.33±0.57^{a}	4.66±0.55^{a}	7.30±0.10^{a}	3.93±0.15^{a}	1.01±0.01^{a}	0.29±0.15^{a}	41.66±0.50^{a}
AB1	87.33±1.15^{b}	9.33±0.55^{b}	9.90±0.20^{b}	5.13±0.05^{b}	1.60±0.01^{b}	0.40±0.01^{b}	44.00±0.50^{b}
AB3	90.66±0.57^{c}	11.33±0.55^{b}	11.16±0.10^{c}	6.16±0.01^{c}	1.87±0.01^{c}	0.53±0.02^{c}	49.33±0.50^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 22: *In-vivo* study of isolates on growth of *Abrus* plant

A. Seed germination and morphological parameters

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)
Control	70.66±0.55^{a}	8.67±0.55^{a}	15.50±0.30^{a}	5.36±0.05^{a}	2.73±0.05^{a}	1.20±0.02^{a}
AB1	83.33±1.00^{b}	13.33±0.25^{b}	17.10±0.10^{b}	6.23±0.05^{b}	3.16±0.05^{b}	1.70±0.02^{b}
AB3	89.33±1.15^{c}	17.33±0.55^{c}	18.40±0.10^{c}	7.40±0.05^{c}	3.83±0.05^{c}	2.20±0.01^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

B. Biochemical parameters

Strain	Antioxidant Activity (%)	Total Carbohydrate (mg/L)	Chlorophyll Content (mg/L)	Flavonoid Content (%)	Nitrate (mg/L)	Total Protein (mg/L)
Control	59.33±0.25^{a}	91.76±0.02^{a}	41.33±0.25^{a}	29.13±0.01^{a}	0.22±0.01^{a}	0.28±0.03^{a}
AB1	63.66±0.35^{b}	94.80±0.03^{b}	44.66±0.25^{b}	31.13±0.01^{b}	0.60±0.01^{b}	0.39±0.03^{b}
AB3	68.00±0.25^{c}	98.16±0.03^{c}	48.33±0.25^{c}	33.13±0.01^{c}	0.65±0.01^{c}	0.42±0.03^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 23: *In-vitro* study of isolates on growth of *Clitoria* plant

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)	Chlorophyll (mg/L)
Control	81.33±0.55^{a}	6.66±1.15^{a}	7.53±0.15^{a}	3.40±0.20^{a}	0.90±0.01^{a}	0.30±0.01^{a}	40.33±0.30^{a}
ClU1	86.66±0.55^{b}	8.66±1.15^{ab}	9.20±0.30^{b}	4.10±0.10^{b}	1.11±0.01^{b}	0.44±0.01^{b}	44.00±0.30^{b}
ClU2	89.33±0.55^{c}	10.66±1.15^{c}	10.20±0.15^{c}	4.46±0.10^{c}	1.20±0.01^{c}	0.50±0.01^{c}	45.32±0.30^{b}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 24: *In-vivo* study of isolates on growth of *Clitoria* plant

A. Seed germination and morphological parameters

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)
Control	79.33±0.25^{a}	7.33±0.55^{a}	17.30±0.10^{a}	7.23±0.10^{a}	2.40±0.02^{a}	1.13±0.01^{a}
ClU1	85.33±0.25^{b}	13.33±0.55^{b}	21.30±0.10^{b}	9.10±0.10^{b}	4.10±0.01^{b}	1.92±0.01^{b}
ClU2	88.66±0.25^{c}	17.33±0.55^{c}	22.50±0.10^{c}	10.20±.10^{c}	4.33±0.01^{b}	2.08±0.01^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 level

B. Biochemical parameters

Strain	Antioxidant Activity (%)	Total Carbohydrate (mg/L)	Chlorophyll Content (mg/L)	Flavonoid Content (%)	Nitrate (mg/L)	Total Protein (mg/L)
Control	55.33±0.25^{a}	94.37±0.03^{a}	41.00±0.02^{a}	27.32±0.05^{a}	0.25±0.02^{a}	0.31±0.05^{a}
ClU1	59.66±0.25^{b}	99.13±0.03^{b}	45.02±0.03^{b}	30.41±0.03^{b}	0.51±0.02^{b}	0.42±0.05^{b}
ClU2	65.66±0.25^{c}	100.30±0.03^{c}	47.66±0.02^{c}	31.65±0.05^{c}	0.59±0.02^{c}	0.45±0.05^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 25: *In-vitro* study of isolates on growth of *Crotalaria* plant

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll (mg/L)
Control	91.33±0.55^{a}	4.66±1.00^{a}	08.03±0.50^{a}	4.33±0.10^{a}	0.97±0.01^{a}	0.14±0.01^{a}	40.12±0.25^{a}
CV5	97.33±0.55^{b}	8.66±1.00^{b}	10.46±0.20^{b}	6.13±0.10^{b}	1.50±0.01^{b}	0.25±0.01^{b}	48.00±0.20^{b}
CV2	99.33±0.55^{b}	9.33±1.00^{b}	10.96±0.20^{c}	6.86±0.10^{c}	1.60±0.01^{b}	0.27±0.01^{b}	48.33±0.20^{bc}
CGJ	99.33±0.55^{b}	9.33±1.00^{b}	11.40±0.10^{c}	7.20±0.10^{c}	1.64±0.01^{b}	0.28±0.01^{b}	50.13±0.25^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 26: *In-vivo* study of isolates on growth of *Crotalaria* plant

A. Seed germination and morphological parameters

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)
Control	83.33±0.50^{a}	10.66±0.50^{a}	16.76±0.10^{a}	6.56±0.20^{a}	2.21±0.01^{a}	1.03±0.01^{a}
CV5	98.00±0.50^{b}	15.33±0.50^{b}	18.10±0.10^{b}	8.56±0.20^{b}	3.83±0.01^{b}	1.40±0.01^{b}
CV2	98.66±0.50^{b}	16.66±0.50^{b}	19.30±0.10^{c}	9.30±0.20^{c}	4.50±0.01^{c}	2.18±0.01^{c}
CGJ	99.33±0.50^{b}	19.33±0.50^{c}	19.70±0.10^{c}	9.40±0.20^{c}	4.93±0.01^{d}	2.30±0.01^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

B. Biochemical parameters

Strain	Antioxidant Activity (%)	Total Carbohydrate (mg/L)	Chlorophyll Content (mg/L)	Flavonoid Content (%)	Nitrate (mg/L)	Total Protein (mg/L)
Control	64±0.25^{a}	95.82±0.20^{a}	42.0±0.05^{a}	28.05±0.20^{a}	0.22±0.01^{a}	0.31±0.02^{a}
CV2	72±0.25^{b}	98.88±0.20^{b}	46.0±0.05^{b}	30.39±0.20^{b}	0.51±0.01^{b}	0.39±0.02^{b}
CV5	73±0.25^{bc}	99.65±0.20^{c}	50.3±0.05^{c}	31.68±0.10^{c}	0.62±0.01^{c}	0.41±0.02^{c}
CGJ	74±0.25^{c}	100.63±0.20^{d}	52.0±0.05^{c}	32.71±0.10^{d}	0.76±0.01^{d}	0.43±0.02^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 27: *In-vitro* study of isolates on growth of *Leucaena* plant

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)	Chlorophyll (mg/L)
Control	81.33±0.50^{a}	5.33±0.50^{a}	7.50±0.10^{a}	3.53±0.02^{a}	0.94±0.01^{a}	0.24±0.01^{a}	41.33±0.25^{a}
LM	87.33±0.50^{b}	6.66±0.50^{ab}	9.16±0.10^{b}	4.16±0.02^{b}	1.22±0.01^{b}	0.29±0.01^{b}	43.66±0.25^{b}
LN	89.33±0.50^{bc}	7.33±0.50^{ab}	9.43±0.10^{c}	4.40±0.02^{c}	1.34±0.01^{c}	0.32±0.01^{c}	45.33±0.25^{c}
LB2	91.33±0.50^{c}	8.66±0.50^{b}	10.16±0.10^{d}	4.76±0.02^{d}	1.44±0.01^{d}	0.35±0.01^{d}	48.66±0.25^{d}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 28: *In-vivo* study of isolates on growth of *Leucaena* plant

A. Seed germination and morphological parameters

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)
Control	78.66±0.50^{a}	12.66±0.50^{a}	15.36±0.05^{a}	5.13±0.02^{a}	2.00±0.01^{a}	0.91±0.01^{a}
LM	85.33±0.50^{b}	14.66±0.50^{a}	18.30±0.05^{b}	7.26±0.02^{b}	3.20±0.01^{b}	1.45±0.01^{b}
LN	85.33±0.50^{b}	17.33±0.50^{b}	19.20±0.05^{c}	7.60±0.02^{c}	3.53±0.01^{c}	1.50±0.01^{c}
LB2	90.66±0.50^{c}	19.33±0.50^{b}	20.22±0.05^{d}	8.70±0.02^{d}	3.80±0.01^{d}	1.55±0.01^{d}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

B. Biochemical parameters

Strain	Antioxidant Activity (%)	Total Carbohydrate (mg/L)	Chlorophyll Content (mg/L)	Flavonoid Content (%)	Nitrate (mg/L)	Total Protein (mg/L)
Control	52.33±0.20^{a}	95.23±0.30^{a}	42.33±0.20^{a}	26.22±0.01^{a}	0.24±0.02^{a}	0.30±0.10^{a}
LM	61.33±0.25^{b}	99.42±0.30^{b}	45.66±0.20^{b}	29.23±0.01^{b}	0.62±0.20^{b}	0.40±0.10^{b}
LN	62.66±0.20^{c}	99.65±0.30^{c}	47.33±0.20^{c}	29.87±0.01^{c}	0.64±0.02^{b}	0.43±0.10^{c}
LB2	65.66±0.20^{d}	100.23±0.30^{d}	49.33±0.20^{d}	30.13±0.01^{d}	0.67±0.02^{c}	0.46±0.01^{d}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 29: *In-vitro* study of isolates on growth of *Sesbania* plant

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)	Chlorophyll (mg/L)
Control	93.33±0.50	6.66±0.50	7.4±0.10	3.2±0.10	0.97±0.01	0.26±0.01	42.3±0.20
VYS	99.66±0.50	8.66±0.50	9.2±0.10	5.3±0.10	1.11±0.01	0.45±0.01	47.0±0.20

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

Table 30: *In-vivo* study of isolates on growth of *Sesbania* plant

A. Seed germination and morphological parameters

Strain	Germination (%)	Leaves count	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry weight (g)
Control	95.5±0.50	11.33±0.50	19.1±0.10	6.2±0.20	2.7±0.01	1.03±0.01
VYS	99.5±0.50	17.66±0.50	22.3±0.20	8.2±0.20	4.2±0.01	2.1±0.01

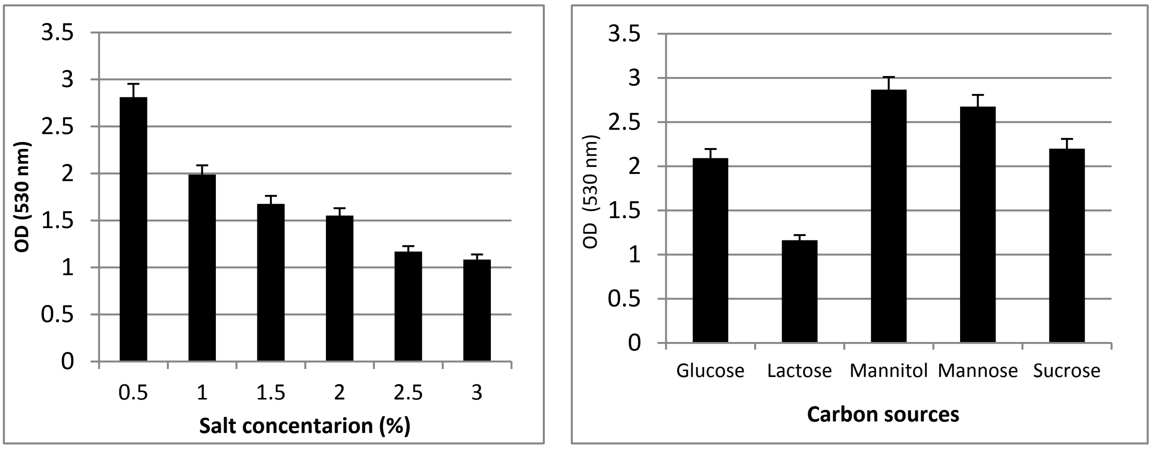
Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

B. Biochemical parameters

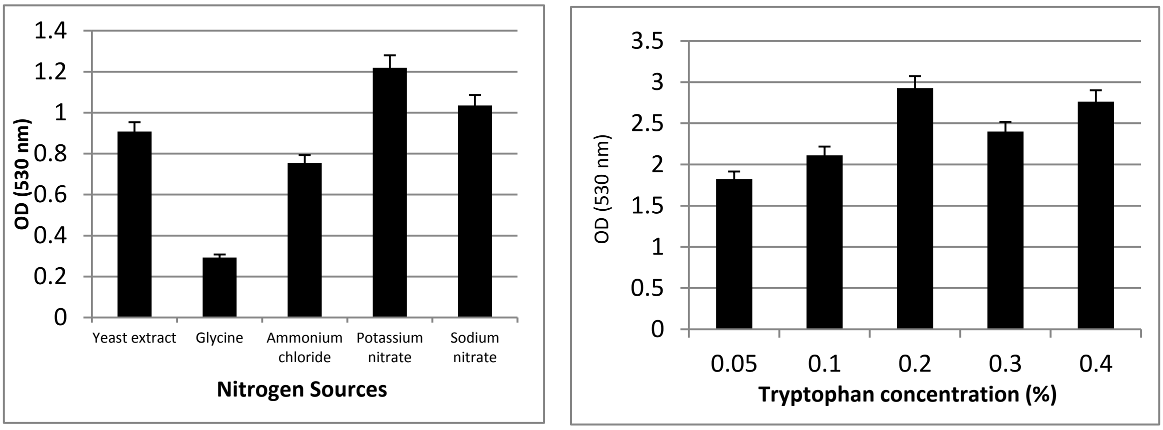
Strain	Antioxidant Activity (%)	Total Carbohydrate (mg/L)	Chlorophyll Content (mg/L)	Flavonoid Content (%)	Nitrate (mg/L)	Total Protein (mg/L)
Control	60.1±0.02	94.32±0.10	43.5±0.10	25.15±0.01	0.24±0.01	0.30±0.01
VYS	63.0±0.02	99.13±0.10	47.5±0.10	29.15±0.01	0.62±0.01	0.41±0.01

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

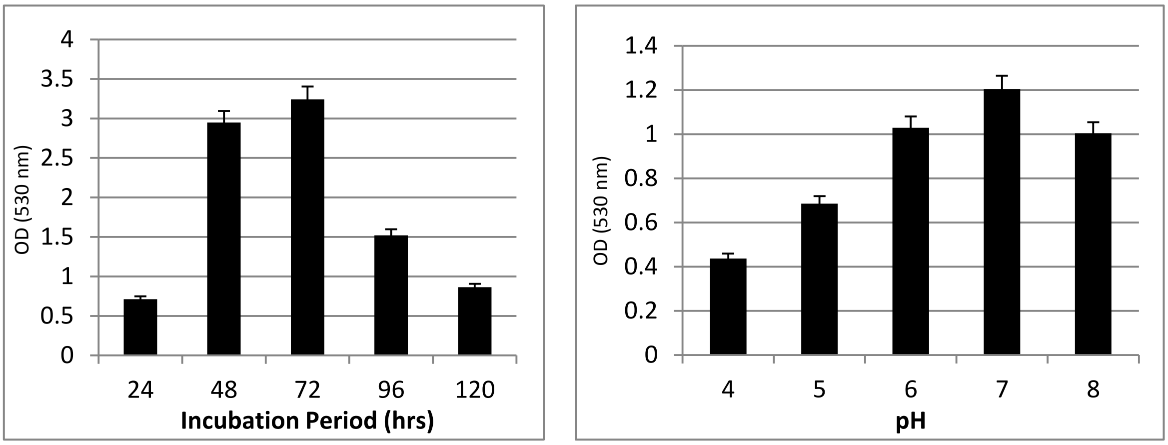
Graph 1: Optimization of IAA production by AB3 under different cultural conditions



a) Different salt concentration **b) Different carbon sources**

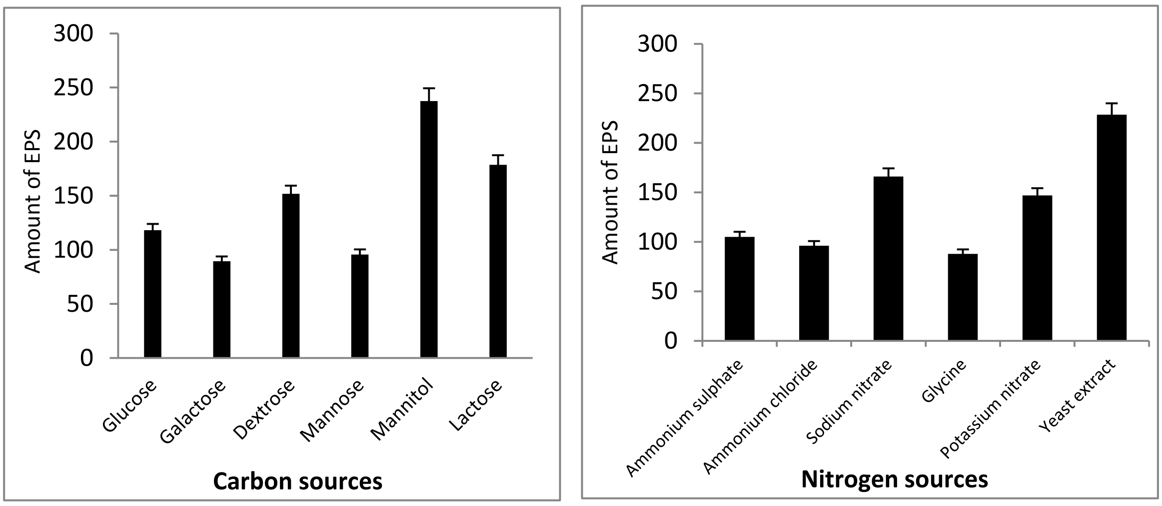


c) Different nitrogen sources **d) Different tryptophan concentrations**



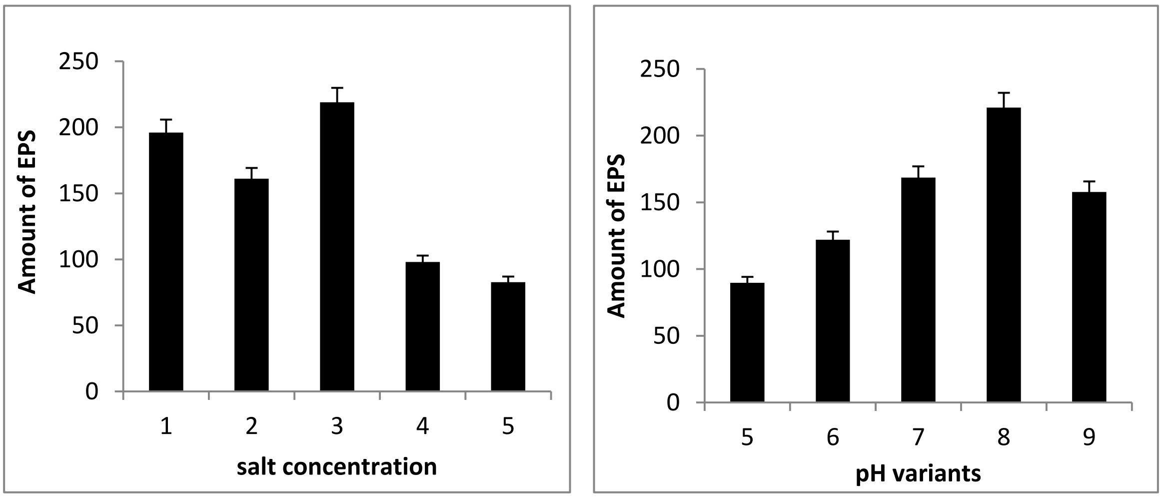
e) Different pH **f) Different incubation period**

Graph 2: Optimization of EPS production by AB3 under different cultural conditions



a) Different carbon sources

b) Different nitrogen sources



c) Different salt concentrations

d) Different pH

DISCUSSION

Discussion

Leguminous plants are economically and ecologically very important and play a very important part amongst globally available biological resources. Legumes have diverse roles in various fields such as agriculture, industries and pharmaceuticals. Legumes are composed of many types of plants including most important traditional folk medicines which mainly belong to the wild habitats (Graham and Vance 2003; Kholina and Voronkova 2012). Wild medicinal legumes contribute in pharmaceutical by being source and raw materials of neutraceuticals and bioactive compounds such as glycosides, antibiotics, alkaloids, flavonoids, and phytochemicals which are used as important components of traditional herbal medicines (Bhat and Karim 2009; Silva et al. 2017). Wild medicinal legumes are more important than cultivated legumes because they are less prone to various environmental stress conditions but due to habitat loss and land use changes these plants are being destroyed without even proper exploration. Hence they need to be conserved which is possible by their proper cultivation and mining for the associated microbes such as root nodulating bacteria.

Density and diversity of legumes is high in tropical and subtropical parts of the world. India is geographically and ecologically very diverse country and have rich assortment of wild medicinal legumes. Sen and Chakraborty (2017) reported that on their revival and modernization, traditional herbal medicines of India have important future in various clinical practices for integrated disease management. Wild medicinal plants are able to grow in diverse types of ecosystems. Although occupancy of these plants in India are higher in hill regions, mainly Himalayan belts but plains of the country are also have to various medicinal plants. Lucknow is a metropolitan city in the state of Uttar Pradesh and it also has some forest area. According to India State of Forest report (2011) forest cover of

Lucknow is 11.91 %. In the present study the forest and uncultivated land around Lucknow and Kanpur region were explored for wild medicinal legumes and their root nodulating bacteria. In this study five wild medicinal legumes *Abrus, Clitoria, Crotalaria, Leucaena* and *Sesbania* were collected from diverse regions. Nodules collected from legumes were of diverse types on the basis of morphology, which is the most noticeable trait in legume-rhizobia symbiosis (Doyle 2011). Formation of nodule types is dependent on the host plant not on the specific rhizobia (Oono et al. 2010). Nodulation (nodule number, type, and structure) of five wild medicinal legumes (*Abrus*, *Clitoria, Crotalaria, Leucaena and Sesbania*) was observed. These wild legumes have shown a great variation in nodulation in respect to site or locality.

Current study deals with the identification, characterization and diversity analysis (morphological, physiological, biochemical, functional, symbiotic and genetic diversity) of bacterial symbionts isolated from root nodule of collected wild medicinal leguminous plants. All the isolates were characterized by polyphasic taxonomy which depends on the phenotypic and genotypic characters (Niemann et al. 1997; Berrada and Fikri-Benbrahim 2014). All the isolates were grown on YEMA media and formed convex, mucilaginous colonies with sooth margins (Vincent 1970). On CRYEMA media most of the isolates formed white mucilaginous colonies (not absorbed congo red dye) and few isolates formed pink or red colonies (absorbed congo red dye) (Somasegaran and Hoben 1985; Jida and Asefa 2011). According to Somasegaran and Hoben (1994) the inability of the isolates to absorb Congo red dye is a distinctive character of rhizobia. However, exceptionally some strains of rhizobia can absorb Congo red which is depends on age of culture, concentration of the dye and exposure to light (Somasegaran and Hoben 1985) to produce orange or deep pink colonies, for example, species of *R. radiobacter* and *Burkholderia* (Howieson and Dilworth 2016). Pseudo-nodule forming bacteria *Agrobacterium* utilize Congo red but

Rhizobium strains do not utilize congo red (Murugesen et al. 2010). This test is essential to differentiate *Rhizobium* and *Agrobacterium* (Deshwal and Chaubey 2014). In general rhizobia absorb the dye weakly whereas many other bacteria take it up strongly. Congo red absorption does not distinguish rhizobia from other bacteria but may be useful as a strain marker i.e. intensity of absorption of dye depends on strain of rhizobial isolates (Keen and Larue 1983). Most of the isolates (75%) were fast growing with highly mucilaginous colonies and 2.5-3.5 hrs mean generation time, while rest of the isolates were slow growing less mucilaginous with 7.0-7.5 hrs mean generation time. In other studies it is reported that highly mucilaginous colonies with <4 hrs generation time are fast growers and isolates having generation time >4 hrs are known as slow growers (Fred et al. 1932; Arora et al. 2000; Ansari and Rao 2014). Jida and Asefa (2011) also supported this categorization and according to them fast growers have generation time 1-3 hrs while slow growers have generation time more than 4 hrs. Growth rate of isolates were also determined by BTB test. In this test fast-growing isolates of rhizobia produced yellow colonies with creamy margins within 3 days of incubation and these were acid producers. Slow-growing isolates produced white colonies, but in some cases the colonies were milky and translucent or yellow after 7–10 days of incubation and these slow growers alkalized the medium (Somasegaran and Hoben 1995; Datta et al. 2015). Similarly Sharma et al. (2010) divided rhizobia into fast growers as acid producers and slow growers as alkaline producers.

Bacterial isolates were further characterized by many physiological and biochemical properties. According to Jordon (1984) several phenotypic characteristics have been used to identify root nodulating bacteria of legumes. Rasul et al. (2012) reported that various phenotypic characters such as carbon sources utilization, antibiotic sensitivity, salinity, and maximum growth temperature may be useful for categorization of isolates. Physiological and biochemical characterization of root nodule isolates are known as the basics of polyphasic taxonomy (Berrada and Fikri-Benbrahim 2014). In this study isolates of wild medicinal legumes were identified and characterized by various physiological and biochemical properties and all the isolates were identified as diverse types. Similarly, Ahmad et al. (1984) examined the diversity of rhizobial isolates for their cultural, biochemical, serological and symbiotic characters from wild medicinal legumes (*Macroptilium lathyroides*, *Phaseolus atropurpureus*, *C. ternatea* and *C. verrucosa*) growing in Jamaika. Isolates were characterized by various parameters such as their colony morphology, growth rate, alkali production, tolerance to acid or alkali, type of flagella, temperature sensitivity, resistance to antibiotics, utilization of different carbon sources, serological relatedness and ability to nodulate other host species. Recently similar study is also done by Bhargava et al. (2016) in Tirupati, India and reported the morphological, biochemical and physiological diversity of the rhizobial isolates from various types of wild legumes.

In this study physiology of isolates was determined at various cultural conditions such as carbon and nitrogen source utilization, growth at pH variants, salt concentrations, temperature ranges and also IAR activity. Rhizobial isolates showed significant difference in carbohydrate metabolism and utilization of carbon sources such as glucose, sucrose, lactose, fructose, arabinose and succinate. Carbon source utilization is used as a indicative feature for categorization of rhizobial species (Somasegaran and Hoben 1994; El-Idrissi et al. 1996). Fast and slow growing rhizobia show variable types of carbon source utilization pattern (Hafeez et al. 1995; Mpepereki et al. 1997). In current study most of the isolates were able to utilize most of the applied carbon sources such as mannitol, glucose, dextrose, lactose, galactose, sucrose and maltose. Most of the isolates were fast growing and utilized diverse range of carbon sources as also reported earlier by Linstrom et al. (1988) and De Lajudie et al. (1998). According to Jida and Asefa (2011) fast growing isolates utilize variable range of carbon sources while slow growers utilized limited number of carbon sources. All the isolates were also able to utilize most of the applied nitrogen sources such as yeast extract, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, glutamine and tryptophan. No isolate was able to utilize glycine while most of the isolates were able to utilize other amino acids such as cysteine, lysine and methionine as nitrogen sources. On the basis of this analysis we can say that most of the isolates were able to utilize diverse range of carbon and nitrogen sources for their growth. Most of the isolates were also able to utilized diverse range of nitrogen sources as also reported earlier for rhizobia (Jordan 1984; Bhargava et al. 2016). Optimum temperature for the growth of isolates was 28 ºC but they were also able to grow at temperature range from 15-35 ºC. Similar findings were also reported by Bhargava et al. (2016). In present work all the isolates showed strong tolerance to salinity. All the isolates were able to grow upto 4% salt concentration and 42.5 % isolates were able to grow upto 8% salinity level in growth media. According to Boukhatem et al. (2016) rhizobial isolates from tropical regions are salt tolerant. Tolerance for temperature and salinity can serve as characterization tools for rhizobial isolates (Yadav and Nehra 2013; Pervin et al. 2017). Optimum pH level for the growth of isolates was 7 but some of the isolates were able to grow from pH 4 to 12 (Shetta et al. 2011). All the isolates were able to grow at pH 6-10 and 27.5% isolates were able to grow at pH 4 and pH 12. Many researchers reported the IAR activity as a phenotypic character for discrimination and identification of rhizobial strains of legumes (Miličić et al. 2006; Ansari and Rao 2014; Arora et al. 2018). In this study most of the isolates were sensitive to gentamicin, streptomycin, nalidixic acid, ampicillin, neomycin, erythromycin, and chloramphenicol. However, isolates were resistant to penicillin. Kanouni et al. (2018) also reported the resistance of rhizobia against penicillin. Findings of Ansari and Rao (2014) supported that fast growing isolates were more sensitive to antibiotics such as gentamycin, amikacin, neomycin, tetracycline, kanamycin and streptomycin. Amarger et al. (1997) reported that IAR activity work as diagnostic feature for rhizobial strains.

Biochemical characterization of isolates was done and it was found that all the isolates were positive for catalase, oxidase and nitrate reductase. Similarly Sadowsky et al. (1983) reported that fast-growing rhizobia are positive for catalase, oxidase and nitrate reductase. According to Deshwal and Chaubey (2014) some biochemical characters were also applied as diagnostic properties for rhizobia such as GPA test (Vincent 1970); HAM (Hofer 1935) and lactose test (Bernaerts and De Ley 1963). Rhizobial isolates were unable to produce 3-ketolactose in lactose test and efficient growth on GPA media and these characters are known as distinguishing characters of rhizobia (Sayyed et al. 2011). All the isolates showed negative activity for gelatinase and cellulase (Hunter et al. 2007). Most of the isolates were able to grow at 8% potassium nitrate and negative for amylase activity (Kumar et al. 2006). According to Deshwal and Chaubey (2014) rhizobia are unable to produce 3-ketolactose in lactose test and grow very well on GPA media and these are the distinguishing characters for them. It is reported that acid production from glucose, production of amylase and catalase, negative test of indole and MR and positive VP and citrate utilization test were very well matched with the characteristics of rhizobial isolates (Sayyed et al. 2011).

Functional diversity of rhizobial isolates were also checked by determining various PGP activities. Bhargava et al. (2016) reported the functional diversity performed by the isolates which can be used for the legume crop production by cross inoculation. This study also determined that most of the isolates were positive for nitrogen fixation, phosphate and zinc solubilisation and production of EPS, IAA, gibberellin and siderophore. Rhizobial isolates are reported as efficient plant growth promoters with various activities such as production of EPS and IAA, phosphate solubilisation and siderophore formation (Laranjo et al. 2014; Vargas et al. 2017). Among all the PGP activities nitrogen fixation is one of the most important activity of rhizobia. Rhizobia are very well reported for their nitrogen fixing activity (Mora et al. 2014; Pérez-Fernández and Alexander 2017). In this study rhizobial isolates were observed as nitrogen fixers and non-rhizobial isolates were also showed this activity which is also reported in earlier studies (Deng et al. 2011; Martínez-Hidalgo and Hirsch 2017). Rhizobia were observed as effective solubilizers of phosphate and zinc and most of the isolates were able to show these PGP traits. Sridevi et al. (2007) and Singh et al. (2014) reported the rhizobial isolates with phosphate solubilisation activity. Vaid et al. (2014) and Kamran et al. (2017) studied the zinc solubilisation property of rhizobia as PGP activity. Only one isolate was positive for HCN production that too with very less activity. Other studies have also reported rhizobia as non-producers of HCN (Antoun et al. 1998; Vidyasekaran and Muthumilan 1999; Manasa et al. 2017). Rhizobial isolates also showed variable range range of siderophore production ability (Arora and Verma 2017). Very few studies report that rhizobia are good producers of siderophores (Arora et al. 2001; Wdowiak-Wro´bel et al. 2017). Quantitative estimation of siderophore production was also estimated by modified microplate method as more efficient procedure (Arora and Verma 2017). In this study among all the isolates one isolate of *Abrus* AB3 identified as most potent plant growth promoter with two main PGP activities EPS and IAA production. These two PGP metabolites were further optimized, extracted and characterized.

IAA is reported as an efficient PGP metabolite produced by rhizobia (Mohite 2013; Othman and Tamini 2016) and more than 74% of rhizobial strains are reported as IAA producers (Halda-Alija 2003). Although, IAA is produced by various PGPR as common product of L-tryptophan metabolism but rhizobia are reported as the dominant group of IAA producing bacteria which promote plant growth (Roy and Basu 2004; Mandal et al. 2007). In this study most of the isolates were able to produce IAA which was estimated by both traditional and microplate method (Sarwar and Kremer 1995; Arora and Verma 2017). Among all the isolates AB3 was identified as most potent IAA producer. It produced maximum amount of IAA (up to 120 µg/ml) while according to Patel et al (2012) only 35.67 µg/ml of IAA was produced by rhizobia isolated from same plant (*A. precatorius*). High efficiency of IAA production can be very effective to promote the plant growth (Etesami et al. 2009; Kumar and Ram 2016). Datta and Basu (2000) reported a very efficient strain of rhizobia producing 99.7 µg/ml IAA. There are various types of environmental factors and incubation parameters (pH, carbon, nitrogen, temperature, and quantities of tryptophan) which affect the synthesis of IAA (Spaepen et al. 2009). At optimum conditions IAA producing ability of bacteria becomes maximum. In this study 0.2% tryptophan concentration was observed as optimum for high production. This is also supported by the results of many other works (Ahmed et al. 2008; Kumar and Ram 2016). IAA production was highest at 72 hrs of incubation, and gradually decreased thereafter. This is also reported by other researchers (Datta and Basu 2000; Sridevi and Mallaiah 2008; Nalini and Rao 2014; Hussein et al. 2016). Neutral pH was recorded as best condition for IAA production by *Rhizobium* sp. like in other reports (Khamna et al. 2010; Madhuri 2011). Mannitol (1%) was found to be best carbon source as reported by other workers which was supported by other works such as Patel et al. (2012) and Ghosh et al. (2015). Potassium nitrate (0.1%) was analysed as best nitrogen source for IAA production. De and Basu (1996) also showed this nitrogen source as best for IAA production by rhizobia. De and Basu (1995) and Ghosh and Basu (1997) used sodium nitrate and potassium nitrate as nitrogen source for IAA production by *Rhizobium* sp. (Mandal et al 2007; Shinde and Patel 2011). Pure IAA was extracted from bacterial culture and purified with TLC. Chromatogram of culture showed a pink spot of purified IAA at the Rf value (0.65) which is almost similar to standard IAA (0.67). Structural analysis of extracted IAA was done by

FTIR spectroscopy (Kamnev et al. 2001; Skoog et al. 2004). In the FTIR spectra, peak observed at wavelength 3286.2 cm^{-1} showed the characteristic (N-H) stretching band of indole moiety which is very similar to the band observed in a study by Sachdev et al. (2009). This is the identifying character of IAA. In spectra (N–H) bending was observed at wavelength 1640.6 cm$^{−1}$. Peak observed at wavelength 2924.7 cm^{-1} and 1460.6 cm$^{−1}$ confirmed the presence of alkyl (–CH2) stretching (symmetric and asymmetric) and bending respectively.

EPS produced by rhizobia also play a very crucial role in the establishment of an effective rhizobia-legume symbiosis (Janczarek et al. 2009; Lehman and Long 2013). EPS is also useful for free-living rhizobia and other bacteria due to several functions such as nutrient gathering, biofilm formation, and protection against environmental stresses to protect bacteria in changing soil conditions (Janczarek 2011; Tewari and Arora 2016). Various studies reported that rhizobia are very potential producers of copious amount of EPS with high viscosity and gel forming ability (Monteiro et al. 2012; Ghosh and Maiti 2016). On the basis of EPS production AB3 was selected and used for further analysis in pure culture conditions. Various environmental factors and specific culture conditions have impact on EPS production such as temperature, pH, carbon sources, nitrogen sources and salt concentrations (Sutherland 1972; De Vuyst et al. 1998; Ghosh et al. 2005). Therefore, it is critically essential to optimize these parameters during the cultivation and synthesis of EPS, which is helpful for the identification of not only novel bio-polysaccharides but also resulting in their higher production (Bomfeti et al. 2011; Castellane et al. 2015). It was observed that the isolate AB3 produced maximum amount of EPS when the media was supplemented with mannitol (1%) as a carbon source and yeast extract (0.1%) as a nitrogen source at 3% salt concentration and slightly alkaline pH. Carbon source in medium is a determining factor for EPS production and mannitol was selected as the most suitable carbon source for AB3 to produce high amount of EPS followed by lactose and this finding was justified by various earlier studies (Nirmala et al. 2011; Liu et al. 2016). Nitrogen source is also important factor for EPS production and in this study yeast extract gave optimum yield of EPS among the different nitrogen source checked also which was reported by Sayyed et al. (2011). It was reported that high level of pH resulted in dramatic decrease in EPS production (Kucuk and Kivanc 2009; Staudt et al. 2012) whereas in this study optimum EPS production was recorded at pH 8.0 as also reported by Sayyed et al. (2011). Salt concentration also affects the EPS production by bacteria (Upadhyay et al. 2011). In present study it was found that 3% salt concentration was best suited for EPS production by AB3. Similar results were also reported by Kanmani et al. (2011) and Sivakumar et al. (2012) that EPS production by bacteria is highest at 2-3% salinity. EPS was extracted from AB3 in form of solid white and water soluble powder (Yadav et al. 2011). In this study 120 μg/ml of EPS was produced by AB3 isolate. Similarly several other studies reported that maximum amount of EPS produced by rhizobia is variable from 116 μg/ml (Mukherjee et al. 2011) to 350 μg/ml (Mandal et al. 2007). EPS was observed as compact crystals with irregular shape and rough surface. Due to the presence of compact structure, extracted EPS can be used in preparation of plastic materials (Sajna et al. 2013). Rhizobial cell produces different types of polysaccharides and they are heteropolymers (Janczarek 2011; Ghosh and Maiti 2016). In FTIR analysis EPS was observed as heteropolysaccharide with relative content of proteins and amino sugars having hydroxyl, carboxyl and amino as main functional groups. The recorded FTIR spectrum showed the characteristic peaks at various wavelengths (3204, 2923, 1672, and 1029 cm-^{1}) which were very similar to peaks reported for typical microbial EPS and proved the nature of biopolymer (Zevenhuizen, 1997; Pal and Paul, 2013; Andhare et al. 2016). The band at 2923-3586 cm^{-1} was assigned as the stretching vibration of hydroxyl group (O-H) which showed the EPS property and water solubility (Kumar et al. 2011; Petera et al. 2015). The peak observed at 2923 cm-^{1} proved the presence of asymmetric vibration of C-H aliphatic group such as CH_2 and CH_3 groups (Fusconi et al. 2010; Pal and Paul, 2013). The peak observed at 1672 cm-^{1} showed the C-O stretching bands indicated the presence of gucosamine (Kumar et al. 2011), succinate and acetate functions also (Andhare et al. 2016). Band observed around the 1322-1438 cm-^{1} could be for carboxylate groups (-COO-) from acid residues such as succinate groups (Andhare et al. 2016). Finally the peaks around 1029 cm-^{1} indicated the presence of glucose as carbohydrate (Delattre et al. 2015). FT-IR spectroscopy could indicate the succinoglycan type of EPS produced by AB3 isolate, which was similar to the results of Andhare et al. (2016). Succinoglycan is a heterogeneous extracellular polysaccharide produced by rhizobia (Bakhtiyari et al. 2015).

Biocontrol activity of isolates was checked against common fungal phytopathgens of legumes. Some rhizobia are known to have efficient biocontrol activity against fungal phytopathogens (Arora et al. 2001; Aeron et al. 2017). In this study isolate LB2 and AB3 were identified as most effective biocontrol agent against *F. moniliforme*, *F. solani* and *F. oxysporum*. LB2 caused maximum inhibition of *F. solani* and *F. oxysporum* while AB3 caused maximum inhibition of *F. moniliforme*. In this regard Dixit et al. (2014) reported rhizobia for biocontrol of *F. oxysporum*. Recently, Kanouni et al. (2018) also reported the inhibitory effect of rhizobia against phytopathogens including *Fusarium* spp.

Nodulation test was done to test the authenticity of isolate as root nodulating bacteria or rhizobia. Nodulation test is very specific for rhizobia and it was observed that only 4 isolates (AB3, LM, LB2 and VYS) showed nodulation test by forming small pink nodules in their respective hosts. All the isolates were not able to form root nodules in other common legumes i.e. cross inoculation was not successful. Similar observations were also reported by Boukhatem et al. (2016) that only four out of eighty one root nodule endophytes successfully caused nodulation in their respective host plants. Sometimes root nodule isolates are not able to cause nodulation in either their host plant or other legumes. It may be possible that some environmental and cultural conditions are not suitable for nodule formation at laboratory conditions.

It is reported that various phenotypic and genotypic methods have been used to identify and characterize rhizobial isolates since many years (Vincent 1970; Obaton et al. 2002). Phenotypic methods are useful for rhizobial identification but molecular methods are more reliable and authentic to study the bacterial identification and diversity analysis. In this regard 16S rRNA gene sequencing is very important technique for identification of bacterial isolates upto their species level. Janda and Abbott (2007) reported that 16S rRNA sequence analyses allowed better identification of bacteria at species level. In this study 11 selected PGP bacterial isolates (on the basis of PGP characters) were characterized by 16S rRNA sequencing. Phylogenetic analysis of sequenced isolates was done to characterize them at species level and to carry out the diversity analysis. The phylogenetic analyses performed in this study confirmed the wide diversity of bacterial isolates associated with wild medicinal legumes. After phylogenetic analysis of the 16S rRNA gene sequencing bacterial isolates were divided into two main groups. One group was of the Rhizobiales (*Rhizobium* and *Beijirinckia*) and second group of the order Enterobacteriales (*Enterobacter, Kosakonia, Cronobacter* and *Pantoea*). Hence, in this study some non-rhizobial isolates were also identified from root nodules of collected wild medicinal legumes. Few other studies also supported the findings of co-existence of rhizobia with diverse non-rhizobial isolates in the root nodules of legumes (Selvakumar et al. 2008; Dixit et al. 2014; Aeron et al. 2015; Sbabou et al. 2016; Lu et al. 2017). Rhizobial isolates were selected and their diversity was analysed by DNA fingerprinting method. RAPD is useful in differentiating between very closely related bacterial isolates. This method is simpler and cheaper and effective for founding genetic relationships (Bardakci 2001; Laguerre et al. 2002). In this study dendrogram derived from RAPD fingerprints revealed that one isolate LB2 differed significantly from all the other strains. The data of RAPD showed considerable diversity in root nodulating bacteria of wild medicinal legumes. Diversity indices of isolates were also calculated and recorded as 2.3-5.2. This value showed the effective diversity among isolates. It is reported that typical diversity values are generally between 1.5 and 3.5 in most ecological studies, and the index is rarely greater than 4. Diversity indices more than 4 indicate a very high level of diversity (Goepel 2012).

Present study reports six bacterial genera *Rhizobium*, *Beijerinckia*, *Enterobacter*, *Kosakonia*, *Cronobacter* and *Pantoea* associated with root nodule of five wild medicinal legumes species growing in Lucknow and adjoining areas. Among eleven genetically identified isolates 5 isolates (AB1, AB3, LM, LB2 and VYS) were found to be of order Rhizobiales, while 6 isolates (ClU1, ClU2, CV2, CV5, CGJ and LN) belonged to order Enterobacteriales (non-rhizobia). Among 5 rhizobial isolates AB3, LM and VYS were identified as *R. pusense*, LB2 as *R. radiobacter* and AB1 as *B. fluminensis. R. pusense* was first time reported by Pandey et al. (2011) from rhizospheric soil of chickpea plant in India and not reported from root nodules of *A. precatorius* or any other legumes in India. In the other countries it has been isolated from root nodules of cowpea (Chidebe et al. 2018) and bean (Aguilar et al. 2016). Out of 6 non-rhizobial isolates, 2 belonged to genus *Kosakonia* (ClU1 as *K. sacchari* and LN as K. *pseudosacchari*), 2 strains belonged to genus *Enterobacter* (ClU2 and CV5 as *E. cloaceae*), one strain CGJ belonged to *Cronobacter sakazakii* and one strain CV2 was *Pantoea agglomerans.* Among all the 5 wild medicinal legumes rhizobia were isolated from only 3 legumes (*Abrus*, *Leucaena* and *Sesbania*) and no rhizobia were isolated from root nodules of *Clitoria* and *Crotalaria*. In this study *K. sacchari* and *K. pseudosacchari* are first time isolated from root nodules of any plants.

These isolates have been reported as endophytes of other plant tissues (Gu et al. 2014; Kampfer et al. 2016) but not from root nodules of any legumes. These isolates are very important for bacterial taxonomy because almost all the isolates were not much explored earlier from root nodules of plants selected in this study and other legumes also either from India or other countries of the world.

This study provided valuable information on the diversity and plant growth promoting attributes of symbiotic bacteria and non-symbiotic bacteria from root nodules of wild medicinal and unexplored legumes from diverse regions. Although such type of study has been done in Himalayan region (Pandey et al. 2006), coastal regions of south Andman (Jayakumar et al. 2010), Rajasthan (Ali et al. 2009) and south India (Tirupati) (Bhargava et al. 2006) for diversity analysis of rhizobial isolates from medicinal legumes, but this is the first study conducted to explore the diversity of root nodulating bacteria associated with wild medicinal leguminous plants growing in Lucknow and adjoining areas. Results clearly show significant rhizobial diversity along with presence of some non-rhizobial genera also from root nodules of medicinal plants included in the study. This study confirms that there is urgent need for the exploration of root nodule associated bacteria from neglected wild medicinal legumes growing in diverse range of ecosystems particularly those which have not been explored so far.

Root nodule bacteria have important PGP characters and some potent rhizobial and non-rhizobial isolates obtained from this study were applied on their respective host plants to check their impact on growth. Rhizobial inoculants are good tool to enhance the yield and quality of legumes in eco-friendly manner without causing any harm to the environments (Arora et al. 2017). Rhizobial inoculants are used for growth promotion of various crops (Arora et al. 2001; Lupwayi et al. 2006; Arora et al. 2017). In this regard Mazhar et al. (2000) reported that inoculation of seedling roots of *Sesbania* spp. by their effective rhizobial isolate enhanced growth, nodulation and nitrogen fixation. Diouf et al. (2003) reported the increased seedling growth of *L. leucocephala* by application of rhizobial inoculants. In this study along with rhizobia some non-rhizobia also identified as efficient PGPR and can be used as effective bioinoculants for higher crop productivity (Aeron et al. 2015; Busby et al. 2016). Rhizobial inoculants not only enhance the plant growth but also increase their nutritional contents which have very potential benefits for human health (Silva et al. 2017). In this study all the selected isolates significantly enhanced the growth of their host plant along with increased bioactive compounds in the plants. Some studies have reported that the inoculation of diverse rhizobial strains in legumes increased the antioxidant potential, flavonoids, alkaloids and protein contents. In this regard Prabha et al. (2013) reported the increased medicinal content and plant growth of on inoculation with fast growing rhizobia. Similarly Irmer et al. (2015) reported the increased nodulation and alkaloid content in *Crotalaria* plant on inoculation with rhizobia. Farfour et al. (2015) reported the increased flavonoid content in faba bean on inoculation with *Rhizobium* as biofertilizer.

Culture collections are valuable resources for the sustainable use of microbial diversity and its conservation. Culture collections impart a vital role in the conservation and sustainable use of microbial resources (Daniel and Prasad 2010). Culture collections also offer the authentic biological material for high quality research in the form of reference strains (Smith 2003). The continued discovery of new microbial taxa and strains need their preservation so as to make them accessible to other researchers and industries. In this regard culture collections play a very important role (Sharma 2014). In this study three useful strains (AB3, LB2 and CV2) were submitted in culture collection centres in India (MCC, MTCC and NBAIM) as type strains and isolate AB3 of *Abrus* plant was assigned with accession number MCC 3409 by MCC, Pune. This strain is taxonomically very important because it is first time isolated from root nodules of a wild medicinal leguminous plant *A. precatorius*. This is also first time that *R. pusense* is being reported from root nodule of any plant in India. MCC accepts microorganisms for deposit under general, safe, patent, and IDA deposit (Sharma 2014). Submission of strain AB3 (MCC 3409) in American Type Culture Collection (ATCC) is also in progress. The information available on rhizobial inoculants and the number of rhizobia stored in different culture collection centres at global level may provide important microbiological resources to increase the use of rhizobial inoculants for sustainable agriculture.

Wild medicinal legumes and their symbionts have increased attention globally due to their higher tolerance to extreme environmental conditions such as drought, salinity and raised temperatures and medicinal value (Zaharan 2001; Dhabhai and Batra 2012). Wild legumes and their associated rhizobia have useful role in providing a vegetational cover in degraded lands and help in reclamation of waste lands (Jha et al. 1995). These legumes also have various agricultural, economic and pharmaceutical importance (Tripathi et al. 2013) but not much exploited and many of its utilizations have not been well excavated. Although various research efforts have been done to improve their productivity but not done with much success. These wild plants are very suitable for present day situation of climate change due to their resilient properties. But due to increased habitat loss and over exploitation there is major loss of such plants around the globe. There is very urgent need of their conservation and protection. This study provides the basis for further research on the phylogeny and biodiversity of rhizobial strains as well as non-rhizobial strains of wild medicinal legumes of in central Uttar Pradesh. Still a large number of legumes are yet to be studied but the legume plants used in this study are very less explored and generally neglected. This study also utilizes the bioinoculants developed from these isolates to improve growth and yield of the medicinal legumes. Inoculation with root nodule isolates is a very reliable agronomic effort to increase the production and conservation of neglected legumes and promotion of sustainable agriculture in order to ensure the food pharmaceutical and economic requirements of the populations along with conservation of important PGPR and reclamation of degraded land.

CONCLUSION

Wild medicinal leguminous plants are not only useful for their medicinal value but these plants also have role in reclamation of waste lands thus they impart big role in sustainable agriculture. The present study provides new information about the phenotypic and genotypic diversity of indigenous rhizobia associated with wild medicinal legumes grown in Lucknow and adjoining areas. We found diverse bacterial strains belonging to rhizobia (*Beijerinckia* and *Rhizobium*) and some non-rhizobia were (*Enterobacter, Kosakonia, Pantoea* and *Cronobacter*) also found from root nodules of selected plants (*Abrus, Clitoria, Crotalaria*, *Leucaena* and *Sesbania*). The positive results of plant growth promoting activities in the isolates indicated their active role in growth enhancement of plants. This work will not only help in knowing about the rhizobial diversity of neglected legumes but also help in conservation of both the plants as well as their bacterial symbionts, which has not been done so far. Application of isolates for growth promotion of their host plant showed potential effects as plant growth promoter. By this work we can explore more diversity of generally neglected wild medicinal legumes for their high medicinal value, which is urgently required. The study also report rhizobial strains for use as biofertilizers and hence lead to organic production of medicinal plants.

SUMMARY

Summary

Leguminosae is one of the largest plant family and leguminous plants are very important due to their outstanding activity of nitrogen fixation in association with their symbiotic root nodulating bacteria. Besides the BNF activity legumes are also source of various beneficial products for human welfare. Legumes produce diverse types of pharmaceutical and bioactive compounds and have a very big role in production of traditional herbal medicines and in this context wild legumes are very important. Besides having medicinal values wild legumes also show resistance against adverse environmental conditions and can not only survive in harsh conditions but can also maintain the fertility of neglected soils. Due to their over exploitation and habitat destruction these plants are now facing extinction. Most of such plants have not been explored for their root nodulating bacteria or commercial production of medicinal metabolites. Mining of rhizobial diversity from wild medicinal legumes growing in waste and uncultivated areas is very important to utilize them in pharmaceutics and reclaim degraded lands.

In this study 40 root nodulating bacteria were isolated from root nodules of five wild medicinal legumes (*Abrus, Clitoria, Crotalaria, Leucaena* and *Sesbania*) collected from different uncultivated sites of Lucknow and adjoining areas (Barabanki, Unnao, Kanpur). All the isolates formed convex, mucilaginous colonies with smooth margins on YEMA and CRYEMA media. All the isolates were identified as Gram negative, motile and rod shaped bacteria. Most of the isolates (75%) formed highly mucilaginous colonies and were fast growers. Of all, and 25% isolates were less mucilaginous and slow growers. Physiologically all the isolates were very diverse types and able to grow under various cultural conditions. Most of the isolates were able to grow at diverse range of pH (5-12), temperature (15-35 ºC) and salinity (1-8%) and were able to utilize various carbon and nitrogen sources. On the basis of IAR activity large number of the isolates was sensitive to most of the antibiotics except penicillin. Biochemically all the isolates were very diverse types. All the forty isolates were positive for catalase, oxidase, nitrate reductase and ammonia production ability while none of the isolates showed gelatinase and cellulase activity. Most of the isolates, 62.5% and 72.5%, showed positive growth on GPA and potassium nitrate (8%) supplemented media, respectively. Among all the isolates 27.5% were able to grow on HAM media and showed positive lactose test. Most of the isolates, 80% and 77.5%, were positive for citrate utilization and PHB production respectively. Among all the isolates 57.5% were positive for urease activity, 62.5% for MR test and 37.5% for VP test. Some enzymatic activity such as amylase, protease and lipase were also showed by 67.5% 42.5% and 2.5% isolates respectively.

PGP nature of all the isolates was also determined by analysing activities, both qualitatively and quantitatively. Most of the isolates were positive for various PGP activities such as nitrogen fixation (97.5% positive), phosphate solubilisation (80% positive), zinc solubilzation (70% positive), gibberellin production (60% positive), IAA production (60% positive), HCN production (2.5% positive), siderophore production (50% positive) and EPS production (100% positive). However, only 2.5% isolates were positive for HCN production. On the basis of biocontrol activity it was observed that amongst all, 35% showed antagonism against *F. moniliforme*, 15% against *F. solani* and 10% against *F. oxysporum*. Of the forty isolates 11 were selected on the basis of PGP characteristics for further studies. Amongst these AB3 was identified as the most potent plant growth promoter with highest level of IAA and EPS production abilities.

IAA production is very important PGP trait of rhizobia. In this study AB3 was identified as efficient IAA producer with 120 µg/ml of IAA production under optimized cultural conditions which was quantitatively estimated by both traditional and microplate method. It was found that AB3 produced maximum amount of IAA when growth medium was supplemented with 0.2% tryptophan, 0.5% salt concentration, mannitol (1%) as carbon source, potassium nitrate (0.1%) as nitrogen source at neutral pH for 72 hrs of incubation. At these optimized growth conditions IAA was produced and extracted. Extracted IAA was purified by thin layer chromatography (TLC) and chromatogram of culture showed a pink spot of purified IAA at the Rf value (0.65) almost same to standard IAA (0.67). Extracted IAA sample was further characterized at molecular level by Fourier transform infrared (FTIR) spectroscopy and characteristic indole group was observed. In the FTIR spectra peak observed at wavelength 3286.2 cm^{-1} showed the characteristic (N-H) stretching band of indole moiety. This is the identifying character of IAA.

EPS production is also a very important character of rhizobia and among all the isolates AB3 was identified as highest EPS producer (75 µg/ml). AB3 produced maximum amount of EPS when the media was supplemented with mannitol (1%) as a carbon source and yeast extract (0.1%) as a nitrogen source at 3% salt concentration and slightly alkaline. Under these conditions EPS was produced and extracted as water soluble solid white powder. Structural analysis of isolates was done by SEM, EDS and FTIR. On the basis of SEM analysis, EPS was observed as compact crystalline with irregular shape and rough surface and according to EDS analysis high concentration of calcium, phosphorus and potassium followed by oxygen and carbon was observed with trace amount of magnesium and sulphur. The recorded FTIR spectrum indicated the presence of water solubility with gucosamine, succinate and acetate groups in EPS. In this analysis EPS was observed as heteropolysaccharide with relative content of proteins and amino sugars having hydroxyl, carboxyl and amino as main functional groups.

Nodulation test was done to test the authenticity of isolate as root nodulating bacteria or rhizobia. Rhizobial isolates AB3, LM and VYS showed development of small pink nodules on their respective host *Abrus*, *Leucaena* and *Sesbania* in culture tube under laboratory conditions. These three isolates (AB3, LM and VYS) were also able to nodulate their respective host in pot assay. Isolates were not able to nodulate legumes other than their host plant i.e. cross inoculation were not observed in this experiment. AB3, LM and VYS significantly enhanced the growth and dry weight of respective host in comparison to uninoculated control.

Phenotypic characterization was validated by genotypic analysis which is more authenticating for bacterial identification at species level. By the analysis of 16S rRNA gene sequencing and nucleotide homology, eleven best PGP isolates were characterized genotypically. On the basis of phylogenetic analysis these isolates were identified as strains from diverse genera. The sequence data of all the isolates were submitted to Gen Bank (NCBI) and assigned with respective accession numbers. Among all the eleven isolates five isolates (AB1, AB3, LM, LB2 and VYS) belonged to order rhizobiales, while six isolates (ClU1, ClU2, CV2, CV5, CGJ and LN) belonged to non-rhizobial order Enterobacteriales. Among five rhizobial isolates AB3, LM and VYS were identified as *Rhizobium pusense*, LB2 as *Rhizobium radiobacter* and AB1 as *Beijerinckia fluminensis.* Out of six non-rhizobial isolates two belonged to genus *Kosakonia* (ClU1 as *K. sacchari* and LN as K. *pseudosacchari*), two belonged to genus *Enterobacter* (ClU2 and CV5 as *E. cloaceae*), one (CGJ) to *Cronobacter sakazakii* and one (CV2) belonged to *Pantoea agglomerans.* Among all the five wild medicinal legumes rhizobia were isolated from only 3 legumes (*Abrus, Leucaena and Sesbania*) and no rhizobia was isolated from root nodules of *Clitoria* and *Crotalaria*. These isolates taxonomically have very important role because all of them were not much reported from root nodules of plants used in this study or other legumes also.

Among all the identified strains diversity of selected rhizobia was checked by RAPD. On the basis of dendrogram of RAPD analysis these rhizobial isolates were found to be of three types. AB1, VYS and AB3 belonged to almost similar category and LB2 showed higher diversity among them. Diversity indices among isolates were also calculated in respect to their host plant (4.0) indicating very variability. This diversity index also showed the diversity of wild medicinal legumes collected from different sites. Diversity among eleven genetically identified isolates was high with diversity value 5.2 and diversity among only rhizobial isolates was moderate type true diversity value 2.6.

Eleven selected isolates with potent plant growth promoting abilities were applied (*in vitro* and *in vivo*) on their respective host to check their impact on plant growth. In case of *Abrus* AB1, and AB3, were applied and AB3 was observed as better plant growth promoter than AB1. It was observed that in comparison to control germination rate, root length, shoot length, leaves count, fresh weight and dry weight of AB3 treated plants were significantly increased by 26.42%, 38.05%, 18.70%, 99.88% 40.29% and 83.33% respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of AB3 treated plants were also significantly higher by 14.61%, 6.97%, 16.93%, 13.73%, 195.4% and 50.00% respectively, in comparison to uninoculated plants.

Isolates ClU1 and ClU2 were applied on their host plant *Clitoria* and observed that ClU2 was better plant growth promoter. ClU2 caused higher growth in plants than control and ClU1. It was observed that in comparison to control seed germination rate, root length, shoot length, leaves count, fresh weight and dry weight of ClU2 treated plants were significantly increased by 11.76%, 41.07%, 30.05%, 136.42%, 80.41% and 84.07% respectively. Various properties such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of ClU2 treated plants were also significantly higher by 18.66%, 6.28%, 16.24%, 15.84%, 136.00% and 45.16% respectively, in comparison to control.

Isolate CV2, CV5 and CGJ were applied on their host plant *Crotalaria* and among these three isolates CGJ was observed as best plant growth promoter in comparison to control and other isolates. It was observed that in comparison to control seed germination rate, root length, shoot length and leaves count of CGJ treated plants were significantly increased by 19.20%, 43.29%, 17.54%, 81.33%, 123.07% and 123.30% respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of CGJ treated plants were also significantly higher by 15.62%, 05.01%, 23.80%, 16.61%, 245.45% and 38.70% respectively in comparison to control.

Isolates LM, LN and LB2 were applied on their host plant *Leucaena* and among these three isolate LB2 observed as best plant growth promoter in comparison to control and other isolates. It was observed that in comparison to control seed germination rate, root length, shoot length and leaves count of LB2 treated plants were significantly increased by 15.25%, 69.59%, 31.64%, 52.68%, 90.00% and 70.32% with respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of LB2 treated plants were also significantly higher by 25.47%, 05.25%, 16.53%, 14.91%, 179.16% and 53.33% with respectively in comparison to control.

Isolate VYS was applied on its host plant *Sesbania* and observed as an efficient plant growth promoter which caused significant enhancement in growth in comparison to control In comparison to control seed germination rate, root length, shoot length and leaves count of VYS treated plants were significantly increased by 4.18% , 55.86%, 16.75%, 32.25% 55.55% and 103.88% respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration, flavonoid content, nitrate content and total protein of VYS treated plants were also significantly higher by 04.82%, 05.09%, 09.19%, 15.90%, 158.3% and 36.66% respectively in comparison to control.

Amongst all the isolates three isolates, AB3, LB2 and CV2 selected on the basis of their PGP activities were submitted in three Indian culture collection centres (MCC, Pune, MTCC, Chandigarh and NBAIM, Mau). Culture submission in ATCC is also in progress. Isolate **AB3** of ***Abrus*** was confirmed as type strain ***R. pusense*** and assigned with an accession number **MCC 3409** by MCC Pune, India. In the current study all the isolates from wild medicinal legumes were characterized by polyphasic taxonomy in which properties of isolates were determined phenotypically by morphological, physiological and biochemical characterization. After that nodulation test, functional diversity, genotypic analysis and diversity analysis were done. This work will be very useful for rhizobial taxonomy and with organic production of neglected wild medicinal legumes. This will also help in conservation of both the root nodule bacteria and their host plants by causing higher production at lower costs and less effort in sustainable manner.

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APPENDIX

CONGO RED YEAST EXTRACT MANNITOL AGAR (CRYEMA) AGAR –

Ingredients	**gms/liter**
Dipotassium hydrogen phosphate	0.5
$K_2SO_4.7H_2O$	0.2
Sodium Chloride	0.1
Mannitol	10.0
Yeast extract	1.0
Agar	20.00
Congo red 1% solution	2.5 ml
Distilled water	1000 ml

CZAPEK'S MINERAL SALT-

Ingredients	**gms/liter**
Carboxymethyl cellulose	5.0
Ammonium nitrate	1.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	1.0
Yeast Extract	0.5
Glucose	1.0
Agar	17
Distilled water	1000ml

SIMMON'S CITRATE AGAR –

Ingredients	**gms/liter**
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	20.00
Distilled water	1000 ml

UREA AGAR –

Ingredients	**gms/liter**
Peptone	1.0
Sodium chloride	5.0
Potassium monohydrogen (or dihydrogen phosphate) phosphate	2.0
Glucose*	1.0
Phenol red solution**	6.0 ml
Urea (20%aqueous solution)	100 ml

Agar	20.00
Distilled water	1000 ml

*Add glucose and phenol red to the molten base and steam for 1 hour, cool to 50°C.

**Add 100 ml urea (filter standardized solution) to the basal medium.

TRYPTONE WATER –

Ingredients	**gms/liter**
Tryptone	10.0
Sodium chloride	5.0
Calcium chloride- Previously sterilized $CaCl_2$ was added after autoclaving	1.0ml
Distilled water	1000ml

SKIM MILK AGAR-

Ingredients	**gms/liter**
Skim milk powder	100
Peptone	5.0
Agar	15.0

STARCH AGAR-

Ingredients	**gms/liter**
Starch	20.0
Peptone	5.0
Beef Extract	3.0
Agar	15.0
Distilled water	1000ml

MR-VP BROTH –

Ingredients	**gms/liter**
Peptone	7.0
Potassium phosphate	5.0
Dextrose	5.0
Distilled water	1000.00 ml

TWEEN 80 AGAR-

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5g
Calcium chloride dihydrogen	0.1g
Tween 80	10g
Agar	20
Distilled water	1000ml
Ph	6

GLUCOSE PEPTONE AGAR –

Ingredients	**gms/liter**
Glucose	40
Peptone	5
Agar	20
Distilled water	1000ml

HOFFER’S ALKALINE MEDIUM –

Ingredients	**gms/liter**
Mannitol	10
Dipotassium phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Yeast extract	1
Bromothymol blue	0.016
Distilled water	1000ml

YEAST EXTRACT GLUCOSE AGAR –

Ingredients	**gms/liter**
Glucose	10
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4
Yeast extract	1
Agar	20
Bromothymol blue	25mg

PEPTONE WATER –

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5.0

PIKOVSKAYA’S AGAR –

Ingredients	**gms/liter**
Yeast extract	0.5
Dextrose	10.0
Calcium phosphate	5.0
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Magnesium sulphate	0.0001
Ferrous sulphate	0.0001
Agar	20
Distilled water	1000 ml

WATER AGAR –

Ingredients	gms/liter
Agar	8
Distilled water	1000 ml

STAINS, INDICATORS, AND REAGENTS

a) **Gram staining**

Crystal violet solution: Dissolve 2.0 gm crystal violet in 20.0 m of 95% ethyl alcohol

Gram's Iodine solution: Mixed 1.0 gm Iodine, 2.0 gm potassium iodide in 300.0 ml of distilled water

Safranin: Dissolved 10.0 ml of safranin into 100.0 ml of distilled water

b) **Indole production test**

Kovac'sreagent: Dissolve the 5.0 gm of diaminobenzaldehyde in the 75.0 ml of amyl alcohol. Then add 25.0 ml of hydrochloric acid to the above preparation. Store the reagent in the refrigerator.

c) **MR-VP test**

Methyl red indicator: Dissolve methyl red (0.1 gm) in 500 ml of 95% ethyl alcohol. Add distilled water and filter the preparation.

VP reagent I: 5.0 gm alpha-naphthol was weighed and dissolved in 95.0 ml of absolute ethyl alcohol

VP reagent II: 40% potassium hydroxide (KOH)

d) **IAA production**

Salkowski reagent : 2 ml 0.5M $FeCl_3$ and 49 ml water and 49 ml 70% perchloric acid.

e) **Ammonia production**

Nessler'sreagent : Dissolve 50.0 gm of potassium iodide in 35 ml of distilled water and added saturated solution of mercuric chloride. Added 400 ml of potassium hydroxide. Dilute to 1000 ml by addition of distilled water. Allow to settle for one week. Stored in tightly stopper brown bottles.

f) **HCN production**

2% Sodium carbonate solution: Dissolve 2 gm of $NaCO_3$ in 100 ml of distilled water.0.5% Picric acid solution Mix 0.5 gm picric acid in 100 ml of water.

g) **Amylase production**

Gram's Iodine: 1.0 gm Iodine and 2.9 gm of Potassium iodide was mixed and add water to make total of 300 ml.

h) **Cellulase production**

Congo red dye (1 mg/1 ml solution) is used for staining.

1M NaCl solution is used for destaining.

PUBLICATIONS

Publications

Research Publications:

1) Arora NK, **Verma M** (2017) Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. 3 Biotech 7:381
2) **Verma M**, Verma S, Arora NK (2018) Application of *Rhizobium-Pseudomonas* consortia for enhanced production of mungbean in sustainable manner. Int J Sci Tech Soc 3(2): 54-61
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Book chapters:

1) Arora NK, **Verma M**, Prakash J, Mishra J (2016) Regulation of Biopesticides: Global Concerns and Policies. In: Arora NK, Mehnaz S, Balestrini R (eds) Bioformulations: for Sustainable Agriculture. Springer, India, pp 283-299
2) Arora NK, **Verma M**, Mishra J (2017) Rhizobial bioformulation: Past, present and future. In: Mehnaz S (ed) Rhizotrophs: Plant growth promotion to bioremediation, Springer, Singapore, pp 69-99
4) **Verma M**, Mishra J, Arora NK (2018) Plant growth promoting rhizobacteria: diversity and applications. In: Sobti RC, Arora NK, Kothari R (eds) Environmental Biotechnology: for sustainable future Springer, Singapore

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PROTOCOLS AND METHODS

Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria

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Abstract In this study, siderophore production by various bacteria amongst the plant-growth-promoting rhizobacteria was quantified by a rapid and efficient method. In total, 23 siderophore-producing bacterial isolates/strains were taken to estimate their siderophore-producing ability by the standard method (chrome azurol sulphonate assay) as well as 96 well microplate method. Production of siderophore was estimated in percent siderophore unit by both the methods. It was observed that data obtained by both methods correlated positively with each other proving the correctness of microplate method. By the modified microplate method, siderophore production by several bacterial strains can be estimated both qualitatively and quantitatively at one go, saving time, chemicals, making it very less tedious, and also being cheaper in comparison with the method currently in use. The modified microtiter plate method as proposed here makes it far easier to screen the plant-growth-promoting character of plant-associated bacteria.

Keywords Siderophore · PGPR · CAS assay · Microplate · Plate reader

Introduction

Siderophores are low-molecular weight secondary metabolites with iron-chelating potential. These are compounds with small peptidic molecules having side chains and functional groups which have high-affinity ligand to bind ferric ions and transport them through the cell membrane (Raymond et al. 2015; Niehus et al. 2017). Siderophores are produced by various microorganisms and are classified into four main classes (carboxylate, hydroxamates, catecholates, and mixed type) on the basis of their structural features, functional groups, and types of ligands (Table 1) (Ali and Vidhale 2013; Kumar et al. 2017; Miethke and Marahiel 2007; Aznar et al. 2015). Diverse bacterial and fungal genera ranging from human pathogens to environmental microbes such as plant-growth-promoting rhizobacteria (PGPR) are reported to produce siderophores.

One of the key mechanisms of PGPR in promoting plant growth involves the production of secondary metabolites such as siderophores (Verma et al. 2011; Ghavami et al. 2017). Although iron is abundantly available in soil, most of it is unavailable to the plant or other organisms, because it forms insoluble complexes. Hence, iron deficiency is a major global issue. Siderophores produced by PGPR help in fulfilment of the iron requirement of plants by causing its solubilisation and chelation from organic or inorganic complexes present in soil (Wandersman and Delepelaire 2004; Arora et al. 2013; Singh et al. 2017). Microbial siderophores strongly chelate iron and enhance iron uptake by forming a ferric–siderophore complex even at very low concentrations (Dimkpa et al. 2009; Fernández-Scavino and Pedraza 2013; Boiteau et al. 2016). Siderophores thus not only help in enhancing plant growth, but also play a very important role in providing iron to other organisms including humans. Siderophores produced by PGPR also

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Table 1 Type of siderophores produced by plant-growth-promoting bacteria. Modified from Saha et al. (2015)

S. no.	Siderophore type	Characteristic functional group	Example with microbial source	References
1	Hydroxamate	Esters or acid chlorides or carboxylic acids	Ferrioxamine B–*Pseudomonas fluorescence*	(Maurer et al. 1968); Radhakrishnan et al. (2014)
2	Catecholates	Phenolate or 2,3-dihydroxy benzoate (DHB) binding groups	Enterobactin–*Escherichia coli*	Dave et al. (2006); Grobelak and Hiller (2017)
3	Carboxylates	Hydroxyl carboxylate and carboxylates	Rhizobactin–*Rhizobium meliloti*	Smith and Neilands (1984); Ghavami et al. (2017)
4	Mixed type	Mixture of above mentioned functional groups	Pyoverdine–*Pseudomonas aeruginosa*	Leong and Neilands (1982); Behnsen and Raffatellu (2016)

help in protection of plant from phytopathogens (Arora 2015; Saha et al. 2016). Phytopathogens are inhibited in rhizosphere by siderophore-producing PGPR because of iron starvation or due to competitive exclusion in iron-deficient conditions (Beneduzi et al. 2012; Parmar and Chakraborty 2016; Dalvi and Rakh 2017). Besides plant growth promotion, siderophores also play an important role in bioremediation of heavy metals from contaminated sites by binding to the toxic metals such as Cr^{3+}, Al^{3+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, etc. (Saha et al. 2015). Siderophore-producing microorganisms can thus be used to detoxify heavy metal contamination by mobilization of insoluble heavy metals (Dimkpa et al. 2008; Rajkumar et al. 2010; Hao et al. 2014; Mishra et al. 2017). Siderophore-producing microbes can thus be used in a variety of ways including bioremediation, sustainable agriculture as biosensors, and even in medicine.

Siderophore production ability of microorganisms is commonly detected by the chrome azurol sulphonate (CAS) assay as given by Schwyn and Neilands (1987). For quantitative estimation of siderophore production, supernatants of microbial cultures are used. Solid CAS agar media are also used for detection of siderophore production qualitatively (Raaska et al. 1993). In CAS assay, competition is for iron uptake between the siderophore and ferric complex of the CAS dye (CAS–iron–detergent complex). Siderophore strongly chelate the iron from iron–dye complex and dye becomes free in the media which causes change in colour from blue to orange (Louden et al. 2011).

Quantity of siderophore produced by microorganisms is measured by spectrophotometric estimation. In this traditional method, CAS reagent is mixed with microbial supernatant and amount of siderophore is estimated by taking optical density of each sample individually. However, this method requires large amount of chemical, time, labour, and space. Keeping this in mind, a modified method of siderophore estimation was developed which is far cheaper, time saving, and less laborious. This method of quantitative estimation of siderophore production was developed principally from the classical method of Schwyn and Neilands (1987) using 96 well microtiter plate and plate reader thus enabling the screening of several PGP strains at a time.

Materials and methods

Bacteria and growth conditions

In the present study, 23 siderophore-producing bacterial strains were taken from Culture Collection of Rhizosphere Microbiology Laboratory, Department of Environmental Microbiology, BBA University, Lucknow (Uttar Pradesh, India). Bacterial strains were grown on Luria–Bertani (LB) agar media (Himedia, Mumbai) at 28 °C for 48 h. All strains were preserved in LB slants at 4 °C and in 25% glycerol stock solution at − 80 °C.

Siderophore estimation assay

Bacterial strains were checked for siderophore-producing ability by universal CAS assay (Schwyn and Neilands 1987). Before starting the experiment, glassware was rinsed with 3 mol/l hydrochloric acid (HCl) to remove iron and subsequently washed in deionized water (Cabaj and Kosakowska 2007). Both qualitative and quantitative methods were used to estimate the siderophore production by bacterial strains. For both the methods, CAS reagent was prepared as per Schwyn and Neilands (1987). Briefly, 121 mg CAS was dissolved in 100 ml distilled water and 20 ml of 1 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) solution prepared in 10 mM HCl. This solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution under stirring. HDTMA solution was prepared by mixing 729 mg HDTMA in 400 ml distilled water. The CAS-HDTMA solution was sterilized before further use.

Qualitative method

This assay was performed according to modified method given by Hu and Xu (2011). CAS agar plates were prepared by mixing 100 ml CAS reagent in 900 ml sterilized LB agar medium. Four bacterial strains were spot inoculated on each plate. An un-inoculated plate was taken as control. After inoculation, plates were incubated at 28 °C for 5–7 days and observed for the formation of orange zone around the bacterial colonies (Louden et al. 2011).

Quantitative method

Quantitative estimation of siderophore production by bacterial strain was done by (i) traditional method and (ii) modified microplate method.

Traditional method

Quantitative estimation of siderophore was done by taking supernatant of bacterial cultures grown in LB broth medium (Hu and Xu 2011). For this, 1 ml broth was taken in 1.5 ml centrifuge tube (Thomas Scientific, US) (one for each bacterial culture) and after sterilization inoculated with 10 µl of freshly grown bacterial culture (10^8 colony forming units (cfu) per ml). Four replicates (tubes) were taken for each strain. Apart from this, control tube (un-inoculated broth) was also maintained. After incubation at 28 °C for 48 h, bacterial cultures were centrifuged at 10,000 rpm for 10 min, cell pellets were discarded, and supernatant was used to estimate siderophore. Supernatant (0.5 ml) of each bacterial culture was mixed with 0.5 ml CAS reagent and after 20 min optical density was taken at 630 nm (Spectrophotometer: Thermo Scientific, Evolution 201). Siderophore produced by strains was measured in percent siderophore unit (psu) which was calculated according to the following formula (Payne 1993):

$$\text{Siderophore production (psu)} = \frac{(A_r - A_s) \times 100}{A_r},$$

where A_r = absorbance of reference (CAS solution and un-inoculated broth), and A_s = absorbance of sample (CAS solution and cell-free supernatant of sample).

Modified microplate method

The modified method for estimating siderophore production was carried out using microtiter plate. Supernatant was obtained from 0.5 ml inoculated (5 µl inoculum containing 10^8 cfu/ml) broth in microcentrifuge tube (Thomas Scientific, US). Supernatant (100 µl) of each bacterial culture was added in separate wells of microplate (CLS3474 Sigma) followed by the addition of 100 µl CAS reagent. After incubation, optical density of each sample (placed in wells of microplate) was recorded at 630 nm using microplate reader (Spectra Max M5e). Four replicates were taken for each strain in 96 well plate and siderophore estimated by the same formula as mentioned above.

Correlation analysis between traditional method and modified microplate method

Correlation between the data obtained from both the methods (traditional method and microplate method) was calculated to observe the similarity. Correlation coefficient was checked by software statistical package for the social science (SPSS) (2016) for windows.

Results and discussion

Bacterial strains

The 23 siderophore-producing bacterial strains taken in the study belong to species amongst diverse genera including *Pseudomonas*, *Rhizobium*, *Enterobacter*, *Chronobacter*, *Kosakonia*, *Beijerinckia,* and *Pantoea.* Details of the strains taken in the study with accession number and reference are mentioned in Table 2. All of these bacterial genera and species are well-known PGPR (Farina et al. 2012; Ahemad and Kibret 2014; Majeed et al. 2015; Naqqash et al. 2016) and common inhabitants of rhizosphere. The study also included endophytic strains from family Enterobacteriaceae, namely, *E. cloacae*, *P. agglomerans,* and *C. sakazakii* which are earlier reported to be siderophore producers (Mokracka et al. 2004; Grim et al. 2012; Walpola and Yoon 2013; Pandey et al. 2016). A novel PGPR strain of *Kosakonia pseudosacchari*, which has not been reported to produce siderophore earlier, was also included in this study. The study thus included very diverse PGPR from different locations of plants including rhizosphere, root nodules, and plant tissues (endophyte) (Table 2).

Siderophore estimation

Formation of orange-coloured zone around the bacterial colonies was observed which indicated siderophore production by bacterial strains. It was observed that all the bacterial strains taken in the study were positive for siderophore production. KA19 (*P. aeruginosa*) showed maximum siderophore production ability on CAS agar (Table 3). The production of siderophore was roughly estimated on the basis of size of halo formation on CAS agar. CAS agar method can only give rough idea and is not a perfect method for quantification of siderophore production. Hence, quantitative estimation of siderophore is done using liquid culture media and CAS reagent.

Table 2 Detail of bacterial strains used for siderophore production assay

S. no.	Bacterial strains	Host plant	Collection site	Accession number (Genbank/ MTCC/MCC)	References
1	*P. aeruginosa* (KA19)	*Brassica campestris*	Rhizospheric soil	–	Mishra and Arora (2012a)
2	*P. aeruginosa* (TO3)	–	Rhizospheric soil	FJ685995	Khare and Arora (2010)
3	*Kosakonia pseudosacchari* (LN)	*Leucaena leucocephala*	Root nodule	KY392997	–
4	*P. fluorescence* (JM1)	–	Rhizospheric soil	KT734728	–
5	*Enterobacter cloacae* (CV5)	*Crotalaria juncea*	Root nodule	MF416432	–
6	*Pseudomonas* sp. (NDN1)	*Lycopersicum esclantum*	Rhizospheric region	–	Arora et al. (2008)
7	*P. aeruginosa* (RB1)	*Withania somnifera*	Plant tissue Endophyte	KT761191	–
8	*Kosakonia* sp. (ClU1)	*Clitoria ternatea*	Root nodule	KY392994	–
9	*E. cloacae* (ClU2)	*C. ternatea*	Root nodule	KY178303	–
10	*R. meliloti* (RMP$_3$)	*Mucuna pruriens*	Root nodule	–	Arora et al. (2001)
11	*R. meliloti* (RMP$_5$)	*M. pruriens*	Root nodule	–	Arora et al. (2001)
12	*Rhizobium* sp. (RASH6)	Leguminous plant	Root nodule	–	Singh et al. (2014)
13	*P. fluorescence* (TO7)	*Brassica* sp.	Rhizospheric soil	HQ457044	Mishra and Arora (2012b)
14	*Pantoea agglomerans* (CV2)	*Crotalaria juncea*	Root nodule	KY178304	–
15	*Rhizobium pusense* (LM)	*L. leucocephala*	Root nodule	KY392995	–
16	*R. pusense* (AB3)	*Abrus precatorius*	Root nodule	KY392993 MCC 3409	–
17	*P. tropicalis* (EKi)	*L. esclantum*	Rhizospheric soil	FJ816019 MTCC 9737	Khare et al. (2011)
18	*Cronobacter sakazakii* (CGJ)	*C. juncea*	Root nodule	MF416433	–
19	*P. aeruginosa* (PF07)	*Helianthus annus*	Rhizospheric	–	Tewari and Arora (2014a)
20	*P. aeruginosa* (PF23)	–	Rhizospheric soil	KF598858	Tewari and Arora (2014b)
21	*Beijerinckia fluminensis* (AB1)	*Abrus precatorius*	Root nodule	MF400858	–
22	*Rhizobium radiobacter* (LB2)	*L. leucocephala*	Root nodule	KY392996	–
23	*P. fluorescence* (PF17)	*H. annus*	Rhizospheric soil	KU201600	Tewari and Arora (2016)

In the traditional method, after growth, cell-free supernatant (0.5 ml) is taken for spectrophotometric estimation in cuvette. However, in the proposed method, supernatant (only 100 µl) was poured in wells of microtiter plate. While 0.5 ml CAS reagent was used per tube in the traditional method, only 100 µl was employed in case of microplate method. Thus, there was drastic reduction in the amount of reagents and broth being used. In fact, there is 80% reduction in the requirement for CAS reagent and 50% decrease in the amount of broth used. Apart from this, the proposed method required far less time. As per our calculation in terms of total time required to quantify the siderophore produced (after incubation and centrifugation to get cell-free supernatant), there was 91.7% reduction. Hence, the proposed method is not only more economical, but is also time saving (Table 4). By the 96 well microplate method, siderophore quantification can be done for several strains at one go (Fig. 1). Although other workers have also reported that microplate method is time saving and efficient (Lapinski et al. 1978; Frac et al. 2016), but this is the first report demonstrating the efficiency of 96 well microplate method for siderophore estimation in terms of time, money and being even more efficient than the traditional spectrophotometric method.

Amount of siderophore produced by all the 23 strains was checked and compared for both the traditional and proposed methods so as to determine the efficiency. The absorbance of reference (A_r) or control (un-inoculated

Table 3 Results of siderophore production from bacterial strains and their estimation by qualitative analysis and quantitative analysis (traditional method and modified microplate method)

Bacterial strains	Qualitative analysis	Quantitative analysis		% increase in absorbance by microplate method
		Traditional method (psu)	Microplate method (psu)	
Control		1.12 ± 0.01	1.12 ± 0.01	0.00
KA19	+++	69.16 ± 0.71	69.81 ± 0.16	0.93
TO3	++	41.45 ± 0.44	43.26 ± 0.06	4.18
LN	++	40.44 ± 0.59	41.44 ± 0.09	2.41
JM1	+	24.99 ± 0.60	25.50 ± 0.14	2.00
CV5	+	35.51 ± 0.53	35.77 ± 0.04	0.72
NDN1	+	36.64 ± 0.73	37.35 ± 0.12	1.90
RB1	++	44.43 ± 0.33	44.44 ± 0.16	0.02
ClU1	++	30.39 ± 0.18	30.64 ± 0.10	0.81
ClU2	+	21.46 ± 0.52	21.90 ± 0.15	2.00
RMP3	+	24.12 ± 0.62	24.49 ± 0.07	1.51
RMP5	++	41.12 ± 0.42	41.35 ± 0.05	0.55
RASH6	+	14.13 ± 0.24	14.67 ± 0.04	3.68
TO7	+	22.58 ± 0.60	23.50 ± 0.15	3.91
CV2	+	27.82 ± 0.52	27.90 ± 0.14	0.06
LM	+	12.63 ± 0.15	13.01 ± 0.01	2.92
AB3	+	33.34 ± 0.03	33.61 ± 0.03	0.80
EKi	++	47.19 ± 0.72	47.43 ± 0.18	0.50
CGJ	+	32.55 ± 0.47	33.06 ± 0.15	1.54
PF07	+	27.62 ± 0.37	27.71 ± 0.07	0.32
PF23	++	45.99 ± 0.59	46.33 ± 0.09	0.73
AB1	+	07.97 ± 0.58	08.33 ± 0.08	4.32
LB2	++	45.12 ± 0.05	45.64 ± 0.04	1.13
PF17	++	48.42 ± 0.26	49.54 ± 0.13	2.26

Data are represented by the mean of four replicates ± standard deviation, (+++), high production; (++), medium production; (+), low production

Table 4 Comparative analysis between traditional and microplate methods of siderophore estimation

Comparative analysis	Methods	
	Traditional method	Microplate method
Labour	Requires high labour input	Requires less labour input
Media	96 ml	48 ml
Reagent	48 ml	9.6 ml
(for 23 strains plus control in quadruplicates)		
Accuracy	Less accurate because several samples are handled individually which may cause high handling error	More accurate because several samples (96) can be handled collectively in only one plate which reduces the handling error
Time	180 min (3 h)	15 min

broth and CAS reagent) was significantly similar both in case of traditional and modified microplate method. Concentration of siderophore produced by bacterial strains varied from 7.97 ± 0.58 to 69.16 ± 0.71 psu when measured by the traditional method, while when quantified through the proposed microplate method, it was from

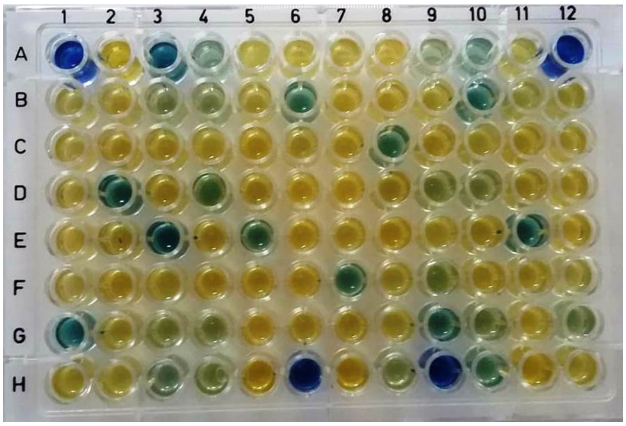


Fig. 1 Estimation of siderophore production by microplate method. Diverse bacterial strains producing different amounts of siderophore

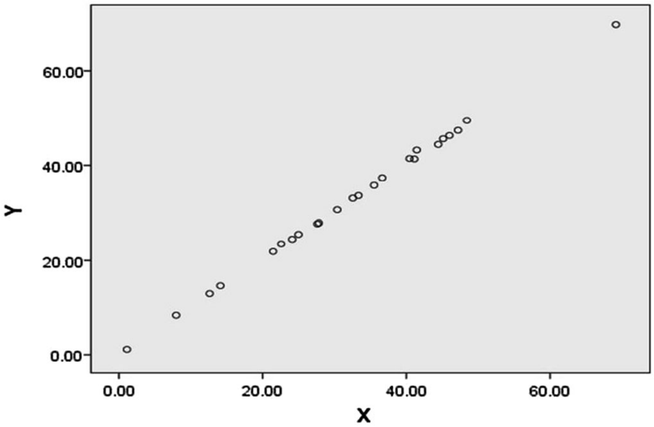
8.33 ± 0.08 to 69.81 ± 0.16 psu (Table 3). Quantitatively also *P. aeruginosa* (KA19) produced maximum amount of siderophore and the readings were significantly similar whether taken by spectrophotometer (traditional method) or by the microplate reader (proposed method). In fact, for all the 23 strains, results were significantly similar whether checked spectrophotometrically or by microplate reader (Table 3). This proves the similarity of both the methods.

Results by both the methods indicated that different strains showed variable siderophore-producing abilities. The present study also proved that fluorescent pseudomonads were the most proficient siderophore producers in comparison with other strains. Many researchers have reported fluorescent pseudomonads to be prolific producers of siderophores (Pandey et al. 2005; Subramanian and Satyan 2014; Pattan et al. 2017; Kotasthane et al. 2017). In fact, *P. aeruginosa* are amongst the most efficient producers of siderophores reported from rhizosphere or other habitats as shown by this work also (de Villegas et al. 2002; Unni et al. 2014; Sasirekha and Srividya 2016). Strains of *P. aeruginosa* (including TO3, RB1, and PF23) were found to be most efficient siderophore producers in comparison with others. Members of the family Rhizobiaceae (except *R. radiobacter* LB2 and *R. meliloti* RMP5) were not very efficient producers of siderophore, and in general, also researchers have not found rhizobia to be prolific producers of siderophores (Joseph et al. 2007; de Souza et al. 2015). Very few studies report that rhizobia are good producers of siderophores (Berraho et al. 1997; Arora et al. 2001; Duhan 2013; Wdowiak-Wróbel et al. 2017). Endophytic strain *K. pseudosacchari* (LN) is being reported for the first time as an efficient siderophore producer. Although production of siderophore is a common phenomenon among PGPR present in rhizosphere (Tewari and Arora 2013; de Souza et al. 2015), recent researches have also shown their production by endophytes residing in the plant tissues and role in plant growth promotion (Zhao et al. 2015; Santoyo et al. 2016; Perez-Rosales et al. 2017).

Correlation analysis between traditional and modified microplate method

Correlation between both the methods was checked so as to measure the similarity between them. It was found that data from both the methods were highly correlated with each other with *R* value of 0.999 (Fig. 2). This is a very strong positive correlation. The value of coefficient of determination (R^2) was 0.999 which indicates that both methods were almost similar in efficiency; however, microplate method is far more rapid and economical. In addition, if the results of quantification by both the methods are observed, it can be seen that microplate method shows slightly higher readings (ranging from 0.02 to 4.32%) in case of all the strains. This proves that microplate method is more accurate in comparison with traditional spectrophotometric method. This may be because absorbance of all the samples was taken at one go using 96 well microplate which reduced the handling error when compared to the traditional method, where absorbance of all the samples is taken

Fig. 2 Correlation between results of siderophore production by two methods: (i) Traditional method = *X* value and (ii) Microplate method = *Y* value



individually. The accuracy factor was further confirmed by small value of standard deviation (SD) in case of microplate method (SD was within 0.18) compared to the traditional method (SD up to 0.73).

Since the classical assay given by Schwyn and Neilands (1987) to check the siderophore activity by bacteria and fungi, some modifications have appeared from time to time (Ames-Gottfred et al. 1989; Milagres et al. 1999; Machuca and Milagres 2003; Pérez-Miranda et al. 2007; Hu and Xu 2011). However, all these modifications were mainly for qualitative analysis only and were not for quantitative estimation. However, here we report a far more economical, time saving, and accurate method for quantitative estimation of siderophore by microbes.

Conclusion

Siderophore production is considered a very important trait of PGPR involved both in growth promotion and in biocontrol of phytopathogens. Siderophore production is also known by other groups of microbes including other soil bacteria and human pathogens. Traditionally, siderophore production and quantification is done by colorimetric/spectrophotometric method. However, in the present study, 96 well microplate method using microplate reader is proposed for estimation of siderophore production by bacteria. The proposed method is far cheaper, consumes less time, and is even more accurate. The suggested method can be used for quantification of siderophore by any bacteria as a better alternative of the routine colorimetric method. Saving chemicals (particularly CAS dye), the proposed method will also be far less harmful to the environment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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RESEARCH ARTICLE

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Application of *Rhizobium-Pseudomonas* consortia for enhanced production of mungbean in sustainable manner

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ABSTRACT

In this study plant growth promoting rhizobacteria (PGPR) were isolated from root nodules of *Vigna radiata* and rhizospheric soil of *Abrus precatorius* respectively. On the basis of morphological, biochemical and physiological characterization, isolate R5 (from root nodules of *V. radiata*) was identified as *Rhizobium* and P21 (from rhizosphere of *A. precatorius*) as *Pseudomonas*. Isolates R5 and P21 showed positive results for phosphate solubilization, zinc solubilization, nitrogen fixation, production of indole acetic acid (IAA), siderophore and exopolysaccharides (EPS). Isolates (R5 and P21) were applied as coinoculant on mungbean (*V. radiata*) to check their impact on its growth in laboratory conditions and pot trials. Seed germination rate and growth analysis of plants were observed by measuring growth parameters (root length, shoot length, leaves count, root nodule count, fresh weight, dry weight and chlorophyll content). It was observed that R5 and P21 significantly enhanced the plant growth in comparison to control (without treatment), but consortial inoculation was more effective in comparison to mono-inoculation of R5 and P21.

INTRODUCTION

Leguminosae is economically and ecologically very important family of Kingdom Plantae (Harborne, 1994). Legumes are the second most important food crops for world agriculture (ILDIS, 2006). Mungbean is a significant pulse crop with high nutritive value and used as a world's major source of food (Yadav *et al.,* 2014). India is the largest producer and consumer of mungbean and accounts for about 65% of the world acreage and 54% of the world production of this crop (Lambrides *et al.*, 2007; Sehrawat *et al.*, 2014). Although mungbean is required on large scale but due to some agro-ecological conditions, the nodulation of mungbean is very poor, which causes lower yields (Ahmed *et al.,* 2006). The excessive uses of chemical fertilizers are used for higher yield, but this approach has several negative implications on our environment (Arora *et al.*, 2012; Hosseini *et al.,* 2014). The application of PGPR as biofertilizers causes a beneficial and cost effective strategy in growth enhancement (Mayak *et al.,* 2004; Mishra and Arora, 2016; Arora *et al.*, 2017).

PGPR impart a great agronomic importance and influence plant development directly by producing metabolites such as plant growth regulators (hormones and other metabolites), siderophore production, phosphate solubilisation and symbiotic nitrogen (N_2) fixation and indirectly through modification to the activity of other plant-microbe interactions or by inducing changes in the microbial population balance, for instance, exerting biological control against plant pathogens (Tewari and Arora, 2013; Ahemad and Kibret, 2014; Vejan *et al.*, 2016; Mishra *et al.,* 2017). The root nodulating bacteria (rhizobia) are well known for their symbiotic association with legumes mainly for biological nitrogen fixation (BNF) (Sessitsch *et al.,* 2002) and used for sustainable crop production (Laranjo *et al.,* 2014; Gopalakrishnan *et al.,* 2015; Arora *et al.,* 2017). One of the most promising groups of bacteria amongst PGPR are *Pseudomonas,* which also have various beneficial plant growth promoting traits (Moeinzadeh *et al.,* 2010; Mayz *et al.,* 2013; Tewari and Arora 2016; Mishra *et al.*, 2017). Currently applications of various PGPR as co-inoculant are growing as a very beneficial trend in sustainable agriculture for higher crop yield.

Co-inoculation of PGPR especially *Pseudomonas* with *Rhizobium* is visualized as an important practice in the development of sustainable agriculture (Singh *et al.,* 2013; Arora *et al.*, 2014). Co-inoculation improves plant growth by affecting some physiological functions such as by reduction in ethylene level (Shaharoona *et al.,* 2006), direct stimulation of rhizobial growth/survival in the soil, enlargement of the root system by hormone production for enhanced nutrient uptake and increase in the number of potential colonization sites by rhizobia (Gull *et al.,* 2004; Barea *et al.,* 2005). This type of co-inoculation is reported to enhance nodulation and N fixation, plant biomass and grain yield in various leguminous crops such as alfalfa (Knight *et al.,* 1988), soybean (Dashti *et al.,* 1997), pea (Kumar *et al.,* 2001), chickpea (Verma *et al.,* 2010), white clover (Arora *et al.,* 2008), galega (Egamberdieva *et al.,* 2010), lentil (Caamano *et al.,* 2018) and common bean (Korir *et al.,* 2017). The seed inoculation with the appropriate PGPR are recommended for higher crop yield since long time, but recently the co-inoculation of N_2 fixing rhizobia and PGPR with diverse characters is emerging as a popular approach rather than inoculating a single organism (Khanna *et al.,* 2011). The aim of this study was to check the plant growth promoting activities of bacterial isolates (*Rhizobium* and *Pseudomonas*) and their co-application as consortia to enhance the growth of mungbean.

MATERIALS AND METHODS

1) Sample collection and isolation of bacteria:

The intact root system bearing nodules of *V. radiata* plant were uprooted carefully and after cutting off the shoot portion plants were aseptically placed in plastic/ polypropylene bags, and immediately brought to laboratory for isolation of rhizobia. Rhizospheric soil of *A. precatorius* plant was collected and used for isolation of PGPR. Sites for sample collection was within the campus of Babasaheb Bhimrao Ambedkar University, Lucknow (26.8467° N, 80.9462° E). Rhizobial isolate R5 was isolated and grown on yeast extract mannitol agar (YEMA) media (Hi Media, Mumbai) (Vincent, 1970). Isolate was also preserved on YEMA slants and in 25% glycerol stock solution at -80 °C. Isolate P21 from rhizospheric soil was isolated and grown on King's B agar media (King *et al.,* 1954) (Hi Media, Mumbai). Colonies were purified and preserved on King's B agar slants and in 25% glycerol stock solution at -80 °C.

2) Characterizations of isolates

The bacterial isolates (R5 and P21) were characterized by various morphological, biochemical and physiological tests according to Bergey's Manual of Systemic Bacteriology (Garrity *et al.,* 2005). Both the isolates were checked for nodulation on mungbean by tube assay.

3) Plant growth promoting characteristics of isolates:

Plant growth promoting potential of isolates was determined by various tests:

a) Phosphate solubilisation

This assay was done on plates containing Pikovskaya's agar media by spot inoculation method (Pikovskaya, 1948). After inoculation plates were incubated at 28 °C for 48 hrs and observed for halo formation around the bacterial colonies.

b) Zinc (Zn) solubilzation

For this, Zn solubilising basal agar medium containing 0.1% insoluble zinc sources such as zinc oxide (ZnO), zinc carbonate ($ZnCO_3$) and zinc phosphate ($Zn_3(PO4)_2$) was prepared and spot inoculated with bacterial culture (Fasim *et al.,* 2002). After inoculation plates were incubated at 28±1°C for 3 days and observed for the formation of clear zone around the colonies.

c) Siderophore production

The detection of siderophore production was done on chrome-azurol sulfonate (CAS) agar media (Schywn and Neilands, 1987). In this assay CAS agar media plate was spot inoculated with culture, incubated at 28 °C for 4-5 days and observed for the formation of orange coloured zone around the colonies which indicated the positive result for siderophore production. Siderophore production of isolates was also estimated according to the modified microplate method given by Arora and Verma (2017).

d) IAA production

IAA production by bacterial culture was determined by the process of Brick *et al.,* (1991). In this assay tryptophan (0.1%) supplemented broth was prepared and inoculated with bacterial culture followed by incubation at 28±2°C for 5 days. After incubation culture were centrifuged and 1 ml of culture supernatant was mixed with 4 ml of Salkowski's reagent (50 ml 35% perchloric acid + 1 ml 0.5 M ferric chloride solution) and 2 drops of ortho-phosphoric acid and incubated for 20 minutes at room temperature. After incubation development of pink colour showed the positive result.

e) EPS production

EPS production was monitored by chilled ethanol precipitation method (Hong *et al.,* 2002). YEM broth media was prepared, inoculated with a loop full of culture and incubated for 4-5 days. After incubation broth was centrifuged at 8000 rpm at 4°C for 10 minutes. Once the cells get separated the culture supernatant was used for extraction of EPS. EPS was precipitated when supernatant was mixed with double volume of chilled 96% ethanol (2:1).

f) Nitrogen fixing activity

Glucose nitrogen free minimal medium (GNFMM) with BTB (0.0025%) as colour indicator was used for this assay (Nakbanpote *et al.,* 2013). GNFMM plate was inoculated with bacterial culture, incubated for 3-7 days at 30°C and observed the colour change from green to blue around the colonies which indicated the nitrogen fixing ability of isolates.

4) Compatibility test between isolates

Before co-inoculation compatibility between both the isolates was checked by cross streak assay on nutrient agar media plate. In this assay both isolates R5 and P21 were streaked together in form of a single line on nutrient agar plates, incubated for 48 hrs at room temperature and clear zone (if any) along the streaked line was observed at the interaction of the streaks (Anandaraj and Delapierre, 2010).

5) Application of isolates on mungbean

The isolates (R5 and P21) were applied on mungbean as test crop and observed for their impact on plant growth both *in vitro* and *in vivo* conditions. Before bacterial inoculation seeds of mungbean were surface sterilized with 70% ethanol (2 min) and 2% sodium hypochlorite (10 min) followed by 7-8 times washings with sterilized distilled water. Surface sterilized seeds were inoculated with R5 and P21 as following treatments: i) uninoculated seeds as control (T1), ii) treatment with R5 (T2), iii) treatment with P21 (T2) and iv) treatment with both R5and P21 (T4). For bacterial treatments broth of R5 (48 hrs old culture) and P21 (24 hrs old culture) were taken and suspended in 1.0% sterilized carboxymethylcellulose (CMC) solution (Weller and Cook, 1983). For consortial inoculation bacterial culture were equally mixed (1:1) and added to the seeds (Vidhyasekaran and Muthamilan, 1999). Seeds were kept for overnight and air dried before sowing. All the treatments were applied in three replicates. Treated seeds were allowed to germinate on plain water agar media (0.8 %) in glass tubes under laboratory conditions. In each tube 3 seeds were placed. After 2-3 days germination of seeds were observed and recorded. Treated seeds were also planted (5 in each pot) in medium sized plastic pots (24x12x12cm) filled with sterilized soils. The physical properties of experimental soil were also examined (Chapman and Pratt, 1961). The soil characteristics were: pH-8.2, electrical conductivity-1.5ds/m, water holding capacity-55%, organic carbon-0.34%, total nitrogen-973.45Kg/H, total phosphate-11.23Kg/H, soluble potassium-210.34Kg/H and organic matter-0.61%. In laboratory conditions plants growth were observed after 15 days, while in pot trial after 30 days of sowing (DAS). Plants were carefully removed and various growth parameters were recorded such as root length, shoot length, leaves count, root nodule count, fresh weight and dry weight. Chlorophyll content was also analyzed according to Arnon, (1949) by using fresh leaf tissue.

8) Statistical analysis of growth parameters

All the data of plant growth parameters were analysed statistically by analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at 5% level to compare difference between treatment means (Gomez and Gomez 1984). Statistical analysis was done by software statistical package for the social science (SPSS) (2016) for windows.

RESULTS AND DISCUSSION:

1) Isolation and characterizations of isolates

In this study bacterial isolates R5 and P21 were used for coinoculation study. R5 was isolated from root nodules of *V. radiata* plant which formed white mucilaginous colonies on YEMA plate; while P21 from rhizospheric soil of *A. precatorius* formed green fluorescent colonies on King's B medium. R5 formed root nodules on its host *V. radiata.* On the basis of morphological, biochemical and physiological characterizations R5 was identified as *Rhizobium* (Holt *et al.,* 1994) and P21 as *Pseudomonas* (Garrity *et al.,* 2005). The results of biochemical and physiological nature of isolates are mentioned in Tables 1 & 2. Isolate P21 showed fluorescence on King's B medium, was Gram-negative, motile, rod shaped and positive for oxidase and catalase (Garrity *et al.,* 2005). R5 showed distinguishing characters of rhizobia, (Tables 1 and 2) (Deshwal and Chaubey, 2014).

2) Plant Growth Promoting characters of isolates

Both isolates R5 and P21 were observed for various plant growth promoting characters (Table 3). Both isolates were able to solubilize phosphate and zinc showing clear zones around the colonies on Pikovaskya agar plate and zinc supplemented media plates. Results indicated that P21 was more potent for phosphate solubilization while R5 for zinc solubilization. R5 and P21 were able to produce IAA and EPS but R5 was better than P21 for both the attributes. R5 and P21 were able to produce siderophore and P21 was more efficient (siderophore producer) in comparison to R5. Nitrogen fixing ability of isolates was also checked and only R5 was able to fix nitrogen. Rhizobia are very well known for

Table 1. **Morphological and biochemical characterization of bacterial isolates**

Characteristic	R5	P21
Gram staining	-	-
Shape	Rod	Rod
Colour	White	Greenish yellow
Fluorescent green pigment	-	+
Motility	+	+
Citrate	+	-
Urease	-	-
Indole	-	-
MR	+	+
VP	-	-
Catalase	++	+
Amylase	+	-
Protease	-	-
Lipase	-	-
Cellulase	-	++

(-) = negative for the test, (+) = positive for the test (++) = more positive for the test

MR = Methyl red, VP = Voges Proskauer

their outstanding property of BNF (Lupwayi *et al.*, 2004). *Rhizobium* and *Pseudomonas* are well known PGPR and show various plant growth promoting activities such as production of phytohormones, phosphate solubilizing activity and siderophore production (Deshwal *et al.*, 2011; Tewari and Arora 2014; Gopalakrishnan *et al.*, 2015) and are used in bioformulations.

Table 2. **Carbon and nitrogen source utilization pattern of the isolates**

Carbon sources	Isolates		Nitrogen sources	Isolates	
	P21	R5		P21	R5
Mannitol	+	+	Yeast extract	+	+
Glucose	+	+	KNO_3	+	+
Dextrose	+	+	$NaNO_3$	+	+
Lactose	+	+	NH_4Cl	+	+
Galactose	+	+	NH_4F	-	-
Sucrose	+	+	NH_4So_4	+	+
Maltose	+	+	Glycine	-	-

(-) = negative growth, (+) = positive growth

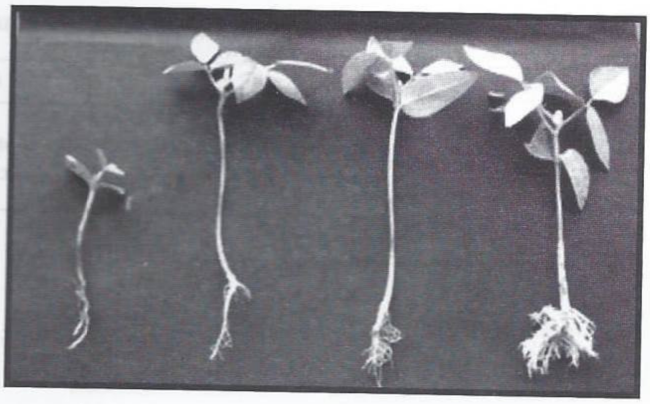

Fig. 1. Effect of bacterial isolates and their consortia on growth of mungbean

3) Compatibility test between isolates:

It was observed that isolates R5 and P21 were compatible with each other and neither of the isolates inhibited each other's growth. Earlier studies have suggested that PGPR strains can be antagonistic or synergistic to each other and it is important to check the compatibility before using them as consortia (Anandaraj and Delapierre, 2010; Singh *et al.*, 2013; Santiago *et al.*, 2017). The compatibility of strains is very crucial for formulating bioinoculants with more than one strain.

4) Application of isolates on mungbean:

R5 and P21 were applied on mungbean alone and in combination. Although R5 (*Rhizobium*) and P21 (*Pseudomonas*) both caused the significant increase in plant growth parameters but co-inoculant (R5 and P21) gave best results (Figure 1). Co-inoculation resulted in significant

Table 3. **Plant growth promoting characterization of bacterial isolates**

S. No.	PGP activity	R5	P21
1.	Phosphate solubilisation	+	+++
2.	IAA production	+++	+
3.	Siderophore production	+	++
4.	Zinc solubilisation	++	+
5.	EPS production	++	+
6.	Nitrogenase assay	++	-

(+) Positive, (++) Good positive, (+++) Excellent positive, (-) Negative

Table 4. **Results of *in vitro* application of isolates on mungbean**

Treatments	Growth Parameters							
	Seed Germination (%)	**Root length (cm)**	**Shoot length (cm)**	**Root Nodule count**	**Fresh Weight (gm)**	**Dry weight (gm)**	**Leaves count**	**Chlorophyll content (mg/L)**
Control (un-inoculated)	81.33±1.15^{a}	3.16±0.05^{a}	8.90±0.01^{a}	-	0.96±0.01^{a}	0.29±0.01^{a}	3.33±1.15^{a}	42.33±0.57^{a}
R5 (*Rhizobium*)	93.33±1.15^{b}	4.52±0.01^{b}	10.85±0.01^{b}	1.66±1.12^{a}	1.25±0.01^{b}	0.45±0.01^{b}	6.66±1.15^{b}	45.00±1.00^{b}
P21 (*Pseudomonas*)	94.66±1.15^{b}	5.45±0.01^{c}	12.95±0.01^{c}	-	1.29±0.01^{c}	0.49±0.01^{c}	7.33±1.15^{b}	47.00±1.00^{c}
R5 + P21 (*Rhizobium*+ *Pseudomonas*)	98.66±1.15^{c}	6.61±0.01^{d}	14.82±0.01^{d}	3.33±1.15^{b}	1.98±0.01^{d}	0.65±0.01^{d}	8.66±1.15^{b}	50.66±0.57^{d}

increase in root length, shoot length, leaves count, root nodule count, fresh weight, dry weight and chlorophyll content of mungbean plants in lab (Table 4) as well as pot conditions (Table 5). It was observed that nodulation was also enhanced by about 30% on co-inoculation in comparison to R5 inoculation alone. The cooperative interaction between rhizobia and other PGPB are found to be very relevant in improving the nodulation as well as nitrogen fixation in legumes (Barea *et al.*, 2005; Figueiredo *et al.*, 2008; Rajendran *et al.*, 2012; El-Nahrawy and Omara 2017). Besides nodulation, co-inoculation between compatible strains can increase plant growth parameters such as root length, shoot length; fresh weight, dry weight, chlorophyll content due to the increased plant nutrition and photosynthesis (Stajkoviæ *et al.*, 2011; Hosseini *et al.*, 2014; Korir *et al.*, 2017). It was observed that root and shoot length of R5 and P21 treated plants were higher with increment of 38% and 20% and 44 and 28% respectively. In comparison to control there was 66 and 46% increase in root and shoot length when both the isolates were applied in combination. Fresh and dry weight of co-inoculated plants was highest (102 and 205% more in comparison to

Table 5. **Results of *in vivo* application of isolates on mungbean:**

Treatments	Growth Parameters							
	Seed Germination (%)	**Root length (cm)**	**Shoot length (cm)**	**Root Nodule count**	**Fresh Weight (gm)**	**Dry weight (gm)**	**Leaves count**	**Chlorophyll content (mg/L)**
Control (uninoculated)	73.70±1.15^{a}	5.01±0.01^{a}	17.26±0.01^{a}	-	3.19±0.01^{a}	1.13±0.01^{a}	7.00±0.57^{a}	42.33±0.57^{a}
R5 (*Rhizobium*)	80.76±0.57^{b}	6.93±0.01^{b}	20.73±0.01^{b}	3.33±0.57^{a}	4.63±0.01^{b}	2.44±0.01^{b}	10.66±0.57^{b}	45.66±0.57^{b}
P21 (*Pseudomonas*)	90.30±1.15^{c}	7.23±0.01^{b}	22.16±0.01^{b}	-	5.14±0.01^{c}	2.54±0.01^{c}	13.33±0.57^{c}	46.66±0.57^{b}
R5 + P21 (*Rhizobium*+ *Pseudomonas*)	99.80±0.57^{d}	8.33±0.01^{c}	25.23±0.01^{c}	4.33±0.57^{b}	6.46±0.01^{d}	3.45±0.01^{d}	17.33±1.00^{d}	50.01±1.00^{c}

Data are represented by the mean of three replicates ± standard deviation; Data followed by the same letters are not significantly different by DMRT at 0.05 levels

control plants. Although individual inoculation of R5 and P21 also increased plant growth parameters significantly less than co-inoculation. Growth enhancement by co-inoculation of rhizobia with other PGPR has been reported as an important method to improve the nitrogen availability in sustainable agricultural practices for other crops (Cambolat *et al.*, 2006; Mishra *et al.*, 2017; El-Nahrawy and Omara 2017).

CONCLUSION

On the basis of this study it is concluded that co-inoculation of *Rhizobium* R5 and *Pseudomonas* P21 showed an increase in the growth and nodulation of *V. radiata*. Application of consortia of both the isolates showed significant increase in germination percentage and biomass over the uninoculated seeds and individual inoculation. Thus co-inoculation of such compatible strains can be used for higher yields of an important pulse crop and lead to sustainable agriculture by replacing chemical fertilizers.

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CHARACTERIZATION AND OPTIMIZATION OF EXOPOLYSACCHARIDES PRODUCED BY RHIZOBIA ISOLATED FROM ROOT NODULES OF MEDICINAL LEGUME *TRIGONELLA* (METHI)

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ABSTRACT

Rhizobia are known as very efficient exopolysaccharide (EPS) producers and EPS plays an important role in various biotechnological applications. In this study twenty (R1-R20) EPS producing rhizobia were isolated from root nodules of medicinal plants named *Trigonella* growing in different areas of Lucknow (U.P.). All the isolates were checked for EPS producing abilities and among all isolates R18 isolate was selected as best EPS producer. The optimization of EPS production by R18 isolate was done using different carbon sources utilization (glucose, galactose, dextrose, mannitol, mannose, lactose, and inositol), different nitrogen sources utilization (ammonium sulphate, ammonium chloride, sodium nitrate, yeast extract, glycine, and potassium nitrate), pH variants (5, 6, 7, 8 and 9) and different salt concentrations (1%, 2%, 3%, 4%, and 5%). Results indicate that isolate R18 showed best growth when medium was amended with mannitol (1%) as carbon source and yeast extract (0.1%) as nitrogen source at pH- 8 and 3% salt concentration. At these optimum conditions isolate R18 was grown and EPS produced by it was extracted by centrifugation process. Extracted EPS was characterized and high amount of sugars were observed. Extracted EPS was also characterized by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and Fourier transform infrared spectroscopy (FTIR) techniques. According to SEM analysis EPS was compact crystalline in nature with irregular shape and rough surface. EDS analysis showed that in EPS the concentration of Ca, P, K were highest followed by O, C, Mg and S were also present in trace amounts. The FTIR spectroscopic analysis was done for functional group analysis of EPS. On the basis of FTIR analysis it was observed that EPS was heteropolysaccharide with relative content of proteins and high amino sugars having hydroxyl, carboxyl and amino as main functional groups.

KEYWORDS: Rhizobia, EPS, *Trigonella*, Medicinal legumes,

INTRODUCTION

Leguminous plants are economically and ecologically very important due to their outstanding nature of biological nitrogen fixation by root nodules formation on host plant. Root nodules are a unique and highly organized structure which is developed by the symbiotic relationship between leguminous plants and bacterial group of the genus rhizobia (Olivares et al., 1984; Gage, 2004). Rhizobia are able to fix atmospheric nitrogen into soil by using the nitrogenase enzyme complex (Kiers et al., 2003). The legume-rhizobia interaction is the result of specific recognition of the host plant by rhizobia through various signal molecules such as flavonoids and Nod factors that are produced specifically by both rhizobia and the legumes (Phillips, 1991). Besides these factors EPS produced by rhizobia also play a very crucial role in the establishment of an effective rhizobia-legume symbiosis (Janczarek et al., 2009; Lehman and Long, 2013). EPS play important roles in various biological processes mainly adhesion and infection, important for the initial stages of the nodules formation (Skorupska et al., 2006). It also protects the cell from desiccation and predation (Bomfeti et al., 2011) and helps in nitrogen fixation by preventing high oxygen tension (Jarman et al., 1978). EPS is also useful for free-living rhizobia and other bacteria due to several functions such as nutrient gathering, biofilm formation, and protection against environmental stresses to adopt bacteria in changing soil conditions (Janczarek, 2011; Tewari and Arora, 2014).

EPS has rapidly emerged as a new and commercially beneficial source of polymeric materials, which are economically competitive with high industrial interest (Singh and Saini, 2012; Ribeiro and Burkert, 2016). Recently exploitation of microorganisms for polysaccharides production has greatly increased due to their novel and unique properties. EPS is non-toxic and biodegradable in nature, hence used as eco-friendly product (Freitas et al., 2011; Donot et al., 2012). EPS molecules are being explored and used as various agents (emulsifiers, stabilizers, binders, coagulants, suspending agents) that lead to numerous commercial applications in diverse field such as bionanotechnology, food, pharmaceutical, cosmetics and various environmental areas (Kumar et al., 2007; Castellane et al., 2015). Microbial EPS can be commercialized in agriculture to deliver various plant growth regulators and biopesticides (Arora and Mishra, 2016; Arora et al., 2016).

Regulation of Biopesticides: Global Concerns and Policies

16

Naveen Kumar Arora, Maya Verma, Jai Prakash,
and Jitendra Mishra

Contents

Abstract

In the last few years, biopesticides have emerged as suitable alternatives to synthetic chemical pesticides. They are cheaper and pose no threat to agroecosystems. This is why their demand and production are also increasing at global level. The law and policies regulating their use and development vary from country to country, and in-depth analysis shows that there is no uniform regulatory model that can simplify their regulation and registration process. Although by the effort of some global agencies such as the International Organization for Biological Control (IOBC), European and Mediterranean Plant Protection Organization (EPPO) and Organization for Economic Co-operative Development (OECD), some flexibility to biopesticide regulation have been provided, but in comparison to chemical pesticides, which have firm market and established nonoverlapping laws, biopesticides lag behind. This chapter provides comprehensive details of regulation systems adopted around the globe and to address shortcomings of existing system; besides this emphasis is also given to adopt innovative practices that could pave way for regulations which are simpler and more universal.

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Rhizobial Bioformulations: Past, Present and Future

4

Naveen Kumar Arora, Maya Verma, and Jitendra Mishra

Abstract

The nitrogen fixing bacterial group known as rhizobia are very important and are used as biological fertilizers for two main purposes; one is to fulfil the nutritional requirements of increasingly populated world and other to overcome the problems arising due to chemical fertilizers. Rhizobial bioformulations are in the market since more than a century and can be the solution for deficiency of nitrogen in our food and soils. Rhizobia maintain the soil fertility along with higher crop yields due to the capability of biological nitrogen fixation (BNF). Currently, various types of rhizobial biofertilizers are commercially available in the market all over the world for agricultural purposes. These can be solid carrier based formulations (organic and inorganic), liquid formulations (with and without additives), synthetic polymer based formulations or metabolite based formulations, but there still is a great room for improvement. However, over the years there have been subtle changes in the rhizobial inoculants in terms of production and application.

4.1 Introduction

Nitrogen (N) is one of the most important nutrients for plant growth. About 1–5 % of total plant dry matter consists of N. It is an essential constituent of proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones and secondary metabolites (Hawkesford et al. 2012). Due to its immense cellular need, N is required in large quantities. Although atmosphere contains 80% of dinitrogen (N_2) (Abd-Alla et al.

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