“Isolation and characterization of endophytic bacteria from Pigeon pea (*Cajanus cajan* L.)”

M.Sc. (Ag) Thesis

By

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“Isolation and characterization of endophytic bacteria from Pigeon pea (*Cajanus cajan* L.)”

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RAJESH KUMAR MAHTO

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CERTIFICATE – I

This is to certify that the thesis entitled **"Isolation and characterization of endophytic bacteria from pigeon pea (*Cajanus cajan* L)."** submitted in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture (Agricultural Microbiology)** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a recorded of the bonafide research work carried out by **Rajesh Kumar Mahto** under my/our guidance and supervision. The subject thesis has been approved by the student's advisory committee and the director of instructions.

No part of the thesis has been submitted for any other degree or diploma or has been published / published part has been fully acknowledged. All the assistance and help received during the course of the investigations have been duly acknowledged by him

Co- Chairman **Chairman**

Date: 2-7-19

THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

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CERTIFICATE II

This is to certify that the thesis entitled **"Isolation and characterization of endophytic bacteria from pigeon pea (*Cajanus cajan* L)."** submitted by **Mr. Rajesh Kumar Mahto** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.) in partial fulfilment of the requirements for the degree of **Master Science in Agriculture (Agricultural Microbiology)** in the **Department of Agricultural Microbiology** has been approved by the external examiner and Student's Advisory Committee after oral examination.

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Director of Instructions _____

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"Education plays fundamental role in personal and social development and teacher plays a fundamental role in imparting education. Teachers have crucial role in preparing young people not only to face the future with confident but also build up it with purpose and responsibility. There is no substitute for teacher pupil relationship". I start in the name of God- who has bestowed upon me all the physical and mental attributes that I possess and skills to cut through and heal a fellow human.

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(Rajesh Kumar Mahto)

Department of Agriculture Microbiology

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Date :

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LIST OF ABBEREVIATION

Abbreviation	Detail
NA	Nutrient agar
NB	Nutrient broth
PDA	Potato dextrose agar
PDB	Potato dextrose broth
MCA	MacConkey
UAB	Urea agar base
SC	Simmon citrate
TSI	Triple sugar iron
MR	Methyl red
VP	Vogues proskeur
MHA	Muller Hinton agar
HCN	Hydro cyanide
CAS	Chrome S Azurol
NSS	Normal saline solution
DNA	Deoxyribonucleic acid
UV	Ultra-Violet
min.	Minutes
hrs.	Hours
c.f.	Centrifuge
rpm	Revolutions per minute
cm	Centimeter
mm	Mili meter
nm	Nanometer
µl	Micro litre
g	Gram
µg	Microgram
µM	Micro mole
l	Litre

M	Molar
%	Percent
EDTA	Ethylene diamine tetra acetic acid
Etbr	Ethidium bromide
Bp	Base pair
16S rDNA	Ribosomal deoxyribonucleic acid
PGPR	Plant growth-promoting rhizobacteria
LAF	Laminar air flow
BOD	Biological oxygen demand
PCR	Polymerase chain reaction

THESIS ABSTRACT

Title of the Thesis	:	Isolation and characterization of endophytic bacteria from Pigeon pea (*Cajanus cajan* L)
Full name of the student	:	Rajesh Kumar Mahto
Major Subject	:	Agril. Microbiology
Name and Address of the Major Advisor	:	Dr. Ravindra Soni, (Assistant Professior) Department of Agriculture Microbiology IGKV, Raipur (C.G.) 492012
Degree to be awarded	:	M.Sc. Ag. (Agricultural Microbiology)

Signature of the student

Signature of Major advisory

Date 2.7.19

Signature of Head of the Department

ABSTRACT

The present studyentitled "Isolation and characterization of endophytic bacteria from pigeon pea" was carried out at ICAR -National Institute of Biotic Stress Management (NIBSM), Baronda, Raipur.A total of 45 bacterial endophyteswere isolated from seven pigeonpea lines and five plant tissues namely root, stem, leaf, flower and pods. The number of isolates isolated tissue wise (19) 42.2%, (11) 24.4%, (11) 24.4%, (2) 4.4% and (2) 4.4% from root, stem, leaf, flower and pod/seed tissues, respectively. Morphological characteristics of bacterial endophytes showed small to medium sized irregular shape colony and the size of most of the isolates varied from pin to medium. Most of the bacterial endophytes (36) have single bacilli and (8) cocco-bacilli, while (1) bacteria had capsulated form. Gram's reaction of bacterial isolates showed that the (28) of bacterial isolates were Gram positive and (17) were gram negative. Biochemical characterization showed that the number of isolates was positive for Oxidase (24), Catalase (30), Citrate

bacterial isolates were Gram positive and (17) were gram negative. Biochemical characterization showed that the number of isolates was positive for Oxidase (24), Catalase (30), Citrate utilization (21), Methyl Red (9) and Vogus Proskauer (3), Urease (10), Nitrate reduction (18). The motility and gases producing ability of isolates showed that 22were motile and 6 isolates produced gas.

Molecular characterization of bacterial endophytes was performed by amplification and sequencing of 16S rDNA gene. On the basis of nucleotide sequences generated by sequencing of 16S rDNA amplicon bacterial isolates were identified and grouped in different genera's namely *Bacillus, Fictibacillus, Pseudomonas*, *Enterobactor, Klebsiella, Beijerinckia, Pantoea,*and *Serretia.* The gene sequences of all the 45 bacterial endophytes have been deposited to Gene bank, NCBI and their accession numbers were obtained.

The bacterial isolates were screened for their plant growth-promoting (PGP) activities viz., production of indole acetic acid (4), siderophore (19), phosphate solubilization (11), DNase (13), HCN production (1) were found positive. Bacterial isolates were screened for antibiotics sensitivity pattern using 14 numbers of multispectral antibiotics showed highest resistance to Ceftriaxone followed by Cifixime, Methicillin, Polymyxin and Ampicilin, while highest sensitivity to Gatifloxacin followed by Gentamicin.

Antagonistic activity test identified bacterial isolates having antagonism against soil borne fungal pathogens *viz, Sclerotium rolfsii* (19) *Fusarium sps* (14) and *Rhizoctonia solani* (17). Highest antagonistic activities against soil borne fungal pathogens were exhibited by bacterial endophytes isolated from root tissues. The potential isolates exhibiting antifungal activities may be used for the development of bio control formulations for controlling multiple biotic stresses.

Key Words: Pigeon pea, Endophytes, Bacteria, Plant Growth Promotion, Antagonistic activities

शोधग्रंथ का सारांश

(अ) शोध का षीर्षक	: अरहर से इंडोफाइटिक बैक्टीरिया का पृथककरण और लक्षण
(ब) छात्र का पूरा नाम	: राजेष कुमार महतो
(स) प्रमुख विषय	: कृषि सूक्ष्मजीव विज्ञान
(द) प्रमुख सलाहकार का नाम व पता	: श्री रविन्द्र सोनी (सहायक प्राध्यापक) कृषि सूक्ष्म जीव विज्ञान विभाग, कृषि महाविद्यालय, रायपुर (छत्तीसगढ़)
(इ) डिग्री से सम्मानित किया जाना हैं	: कृषि स्नातकोत्तर (कृषि सूक्ष्मजीव विज्ञान)

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मुख्य सलाहकार के हस्ताक्षर
दिनांक

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छात्र के हस्ताक्षर

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विभाग प्रमुख के हस्ताक्षर

सारांश

वर्तमान परीक्षण आई.सी.ए.आर. नेषनल इंस्टीट्यूट ऑफ बायोटिक स्ट्रैस मैनेजमेंट (एन. आई.बी.एस.एम.) बरौंडा रायपुर में अरहर के पौधे से इंडोफाइटिक बैक्टीरिया के पृथककरण और लक्षण पर किया गया। कुल 45 इण्डोफाइटिक बैक्टीरिया अरहर के सात लाइन के पांच पादप ऊतको जड़, तना, पत्ती, फूल और फली से पृथक किये गये जिसमें क्रमषः (19) 42.2 %, (11) 24.4% (2) 4.4%(2) 4.4% प्राप्त किये गये। आकारिकीय एवं कलोनी लक्षण जिसमें छोटे से मध्य अनियमित आकार और पिन से मध्यम आकार के बहुत से बैक्टीरिया देखे गये। मुख्यतः 36 बैक्टीरिया ` छड़ आकार के आठ कोकोबैसीलाई और 1 कोकाई जो कैप्सूलेटेड प्राप्त हुए। बैक्टीरिया के ग्राम प्रतिक्रिया जिसमें 28 पाजीटिव और 17 नेगेटिव लक्षण देखे गये। जैव रासानिक परीक्षण जिसमें कुछ संख्या में पाजीटिव पाये गये। जिनमें 12 चलित बैक्टीरिया और 6 गैस उत्पादन करने वाले बैक्टीरिया निकाले गये। न्यूक्लोटाइट के आधार पर विभिन्न

क्लेबसीला, बीजरिंकिया, पोन्टीया और सेरेटिया थे। सभी 45 इण्डोफाटिक बैक्टीरिया जिसका जीन सिकवेंश किये गये और जीन बैंक एन.सी.बी.आई. में जमा कर उनके एक्सेंसन नंबर प्राप्त किये गये। बैक्टीरिया के पादप वृद्धि की सक्रियता को बढ़ाने वाले का स्क्रीन किया गया, जिसमें 4 आई. ए. ए. (इंडोल एसिटिक एसिड) 19 सिडेरोस्फेर 11 फास्फोटेज घुलनशीलता 13 डी. एन. एस. और 1 एच. सी. एन. पाजीटिव पायी गई। 14 मल्टीस्पेक्ट्रम प्रतिजैविक से निकाले गये इण्डोफाइटिक बैक्टीरिया का प्रतिजैविक परीक्षण किया गया जिसमें उच्चरोधक सेप्ट्रियक्सोन के बाद सेफीक्सीन, मेथिसिलीन, पोलीमिक्सीन और एम्पिसिलीन वैसे ही उच्च ग्रहणता में गैटीफ्लोक्सासिन के बाद जेंटामाइसिन देखे गये। बैक्टीरिया का भूमिजनित रोगजनक स्क्लेरोसियम रोल्फसाई, फ्युजेरियम स्पेसीस और रहिजोक्टोनिया से प्रतिरोधक परीक्षण किया गया जिसमें स्क्लेरोसियम रोल्फसाई प्रति 14 फ्युजेरियम स्पेसीस के प्रति 19 और 17 रहिजोक्टोनिया के प्रति प्रतिरोधक सक्रियता प्राप्त हुए। इण्डोफाइटिक बैक्टीरिया का प्रतिरोधक परीक्षण करने पर पायी गई कवक के प्रति उच्चतम प्रतिरोधक सक्रियता मुख्यतः जड़ वाले भाग निकाले गये इंडोफाइटिक बैक्टीरिया से प्राप्त हुए। संभावित एंटिफंगल को प्रदर्शित करता है, कई नियंत्रणों के लिए जैव नियंत्रण के विकास के लिए गतिविधियों का उपयोग किया जा सकता है।

CHAPTER-I
INTRODUCTION

Plants are always involved in communications with a number of microbes including bacterial and fungal communities. These microbes inhabit the rhizosphere, the phyllosphere and the endosphere of plant tissues. These endophyte generally refers to a microbe which colonize the internal tissues of plant without causing any harm or direct symptoms of infection or obvious appearance of disease and live along with plant in mutualistic or symbiotic relationship for at least a part of their life (Kausari and Spiteller, 2012; Kusari *et al.,* 2013). The term endophyte was first coined by de-Bary in the year 1866 as 'endophyte' (*endon* means within; *phyton* means plant). The endophyte includes all the microorganisms that colonize the internal tissues of their hosts and live inside the host for a variable period of their life without causing any disease symptoms (Stone *et al.,* 2000).

Endophytic microflora was first noticed in the 1940's but only at the turn of the 21st century the ubiquity of these microflora was fully recognized (Heinrich, 1997). Furthermore, endophytes are largely unexploited section of microbial biodiversity continuously exposed to intergeneric-genetic exchange with the host plants and in a long run both host and the endophyte coevolves into an asymptomatic relationship. They have a protective role against plant pathogens, insect herbivory and many of them are potential producers of novel antimicrobial secondary metabolites (Arnold and Maynard, 2001).Endophytes are generally protected from stresses and microbial competition present in the surrounding environment by plant harboring endophytes and they appear to be pervasive in tissues, having been isolated from leaf, flower, pod, seeds, stem and root of various plant species (Kobayashi & Palumbo, 2000) and they have several plant growth promoting effects on host plant.

Recently, attention in endophytic microorganisms has increased, as they have an important role in agroclimatic environment and are auspicious because of their impending utilization in sustainable agriculture (Dudeja *et al.,* 2012). The endophytes have been reported in closely 3 lakhs plant species that exist on the earth, each plant harbor more than one endophytic microbes, have been present in practically in all plant studied (Ryan *et al.,* 2008). Associations of these endophytic microbes with plant have been observed to improve plant health and may

benefit them to save from numerous abiotic and biotic stresses (Sapak *et al.,* 2008; Hasegawa *et al.,* 2006).

Pulses are cheap and well-known rich sources of protein, carbohydrates, micronutrients and occupied special position in Indian agriculture. Among them, pigeon pea commonly known as Arhar (*Cajanus cajan* L.) is an important food legume mainly cultivated in tropical and subtropical areas of the world. However, India is the center of origin and largest producer of arhar dal in the world and sharing about seventy percent of the production and also occupies about seventy four percent of the total land area (Bohra *et al.,* 2012). Further, Pigeon pea also have an key role in the food and maintaining nutritional balance of human being, as a good source protein (20-25 %), minerals and vitamins and its various usages in food, fodder, fuel, integrated farming systems, soil conservation and symbiotic nitrogen fixation (Reddy *et al.,* 2005). The importance of *Cajanus cajan* L. as a source of protein and its ability to grow symbiotically on low-N soils and ability of biological nitrogen fixation can help to enhance pigeon pea in sustainable manner.

Endophytic bacteria started to be better analyzed and it became clear that they play a crucial role in providing resistance/tolerance to various biotic and abiotic stresses change in physiological properties, production of phytohormones, enzymes etc., ultimately resulting in higher yields. There are several reports available which showed that in plant-microbe interactions, endophytes pay substances that possess various bioactivities such as antimicrobial and antifungal metabolites. Exploration and exploitation of such plant- microbe (endophyte) interactions can result in the enhancement of plant growth and can play a vital role in low input sustainable agricultural production (Ryan *et al.,* 2007). Plant growth promoting endophytic bacteria facilitate various direct and indirect mechanisms of plant growth promotion. These bacteria are supposed to provoke plant growth by producing phytohormones (like auxin or cytokinin), enzyme ACC deaminase, which drops ethylene levels in plant, indirectly by siderophore production which prevent pathogen infections, or sometime by inducing systemic resistance (ISR) in plant. Other benefits of endophytes to plants include several other PGPR activities like fixation of nitrogen and solubilization of zinc, phosphorous and potassium.

The most important constraint for limiting crop yield is soil infertility, endophytic bacteria known to play an important role in improving soil fertility. These microbes can be deployed for integrated plant nutrient management system through natural resource conservation

and plant growth promoting (PGP) bacteria as a part of mainstream agricultural practices for sustainable agriculture production (Etesami *et al.*, 2014). In recent years, co-inoculation of endophytic microorganism noticed to improve nutrient availability in sustainable agriculture production system and reports indicated that the endophytic bacteria may have the potential to be used as agricultural and microbial inoculants (Compant *et al.*, 2005; Ryan *et al.*, 2008). In addition, they are also known to produce several of bioactive secondary metabolites including steroids, alkaloids, flavonoids, phenolic compounds and enzymes etc. which protect plants against pathogenic invasion (Strobel and Daisy, 2003; Strobel *et al.*, 2004; Banik *et al.*, 2016) that are well-known to produce resistance against many environmental stresses mainly high temperature, salt, disease and drought in plant which harboring endophytes (Stone *et al.*, 2000).

Understanding of the microbial diversity, plant-microbial associations and their function in plant growth is essential to understand the multitude of endophytic relationships with host plants. Understanding of these associations may be helpful in manipulations to enhance agricultural produce, maintain biodiversity and sustainable agriculture in relation to as well as under dry farming circumstances which may benefit in incapacitating abiotic stress. Therefore, host-endophyte relationships need more consideration and investigation in many related aspects such as the multispecies crosstalk and links with herbivores and predators.

Microbial community structure in the unexplored rhizosphere is very important to understand their population dynamics, biology and ecological interaction with the host plant. The diversity of endophytic bacteria in the different parts of pigeon pea plant has specificity of different genotypes and nutrient availability of the tissues. Assessment of endophytic microbial community at particular niche may be beneficial for the plant to combat against biotic and abiotic stresses for nutritional ability. The endophytic community dominance, diversity and stability were diverse depending on the type of plant tissues, stages of plant growth and cultivars. Further, available reports suggested that the endophytes microbes showed community structures across the pigeon pea a higher stability than those of the endophytes diazotrophic bacteria (Rangjaroen *et al*., 2014). To explore the role of endophytes in plants, there should be a better understanding about endophytes, their significance and their importance in plant nutrition, production of novel and effective bioactive compounds and antagonistic effect against the important pathogens.

Very limited information is available on endophytic diversity in the different tissues of pigeonpea. There is an urgent need to identify the tissue specific bacterial endophytes which could be exploited as potential endophytic isolates for plant growth promoting (PGP) traits and controlling biotic stresses in pigeon pea.

Keeping in view the above importance of bacterial endophytes the present study was undertaken on **"Isolation and characterization of endophytic bacteria from Pigeon pea *(Cajanus cajan L.)"*** with the following objectives:

Objective of the study:-

1. To isolate endophytic bacteria from different parts/tissues of pigeon pea.
2. Characterization of isolated endophytes using morphological, biochemical and molecular techniques.
3. *In-vitro* screening of bacterial endophytes for their probable effect in plant growth and management of biotic stress.

CHAPTER-II
REVIEW OF LITERATURE

Consistent with the terminology used for microorganisms colonizing the human body (Qin *et al*., 2010; Gevers *et al.,* 2012), the cooperative groups of plant-associated microorganisms are generally referred as the plant microbiome. This plant microbiome known as a key determinant of plant health and productivity (Berendsen *et al*., 2012) and has received significant attention in recent years (Lebeis *et al*., 2012, Turner *et al.,* 2013). The microbiomes present in internal plant tissues are complex communities of bacteria, archaea and fungi which inhabitant as endophytes across the plants (Turner *et al.,* 2013; Hardoim *et al.,* 2015). Plants are colonized by large number of microbes which can reach much higher cell densities than the number of plant cells (Mendes *et al*., 2013). It is interesting to note that soil microbiomes are now touted as a corner stone of the next green revolution (Parnell 2016). Concept of soil, microbes and plant interface is not new however; the plant microbiome remarks this interaction more along the lines of the 'soil-microbe–soil-plant–microbe-plant interface' rather than the 'soil–microbe–plant interface' Influenced by climatic factors, the rhizosphere in turn, impacts the plant and microbiome that utilizes the habitat as an information highway (Bais *et al*., 2004, Rout *et al.,* 2015). Hence, a brief review of the work reported on endophytic microbes with especial reference to the bacterial endophytes is narrated as follows:

2.1 Definition and history of endophytes

The word endophyte was first given by de-Bary in 1866. It may be a bacteria or fungi, which passes the entire or partial lifespan inhabiting intra or inter-cellularly inside the healthy part of the plant, without showing any ostensible symptoms of illness (Wilson, 1995). The plant microbe relationships in endopshere may begin to evolve from the time when higher plants first appeared on Earth, several millions years ago. Crucial sign of plant concomitant endophytic microorganism has been revealed in the fossilized matters of leaves and stems (Redecker *et al*., 2000). Endophytes, including bacteria and fungi, are ubiquitous microorganisms that live within the host plants without affecting showing any harmful symptoms of the infectious diseases (Sturz *et al*., 2000). They enter inside a plant tissues mainly through the root system, aerial portions like flower, stem and cotyledons also used for mode of entry. Bacterium moves to plant tissue *via* growing radicals, secondary roots, and stomata or through the damages in foliar tissues. After

entry bacteria may exist within the cells in intercellular spaces or in vascular system. They inside the plant may either become localized at the point of entry or spread throughout the plant system. Generally bacterial populations are larger in the roots and decline in the stem and in the leaves. They exhibit mutual association with the plant by producing a wide range of bioactive metabolites (Perotti, 1926; Hallmann *et al*., 1997). They also seem to be involved in plant growth promotional activities including prevention from herbivore attack, plant health, growth and yield (Kloepper and Beauchamp, 1992; Chanway, 1997).

2.2 Endophytes Biodiversity

Microbial groups inhabiting soil play a central and often exceptional role in functioning of ecosystem and are among the most diverse, complex and key assemblages in the biosphere (Zhou *et al*., 2003). It is evident that the bacterial endophytes best adapted for living inside/colonizing the plants/ plant tissues are naturally selected. Endophytic microbes are recruited from a large pool of microbes present in soil or rhizospheric species and clones. Endophytes can actively or latently colonize the plants/ plant tissues locally and/or systemically and both intracellularly and intercellularly. Several reports indicated that the endophytic microbes/ bacteria in a single plant host not restricted to a single species but comprise of several genera and species (Ryan *et al.,* 2008).

2.3 Endophytic bacteria

Endophytes were first reported by the Freeman (1904) from dunel grass. Since then, endophytes are known to occur in higher plants (Storbel *et al.,* 2004). Of the nearly 300000 plant species known to on earth, all are host to one or more endophytes (Strobel and daisy, 2003). All must have endophytes in their tissues, if not, it could be due to the inability to isolate and culture them under laboratory conditions (Rosenblueth and Esperanza, 2006). Bacterial endophytes can easily be isolated from surface-sterilized plant tissue or extracted from inside the plant/tissues through maceration. The relationship between the endophyte and their host, plant / plant tissues may range from latent phytopathogenesis to mutualistic relationship (Sturz *et al.,* 1997).

According to the life cycle strategies, endophytic bacteria can be classified as ‘facultative endophytes’ or ‘obligate endophytes’. Facultative endophyte has a phase in its lifecycle in which it exists outside host plant. Obligates endophytes mainly depends on the host for their growth, development, survival and transmission to other plants through vertically or *via* vectors. In the other hand and extreme cases, bacterial plant pathogen might be included as (obligate or

facultative) endophyte, because they often occur in avirulent form in plants. Avirulent forms of plant pathogens should thus be regarded as entophytes, whereas virulent strains of these organisms should not be included (Hardoim *el al.,* 2008).

Endophytes colonize the leaves, stems, roots, fruits, tubers, ovules, internodal regions and legume nodules (Hallmann *et al.,* 1997; Sturz *et al.,* 1997; Hurek *et al.,* 2002; Benhizia *et al.,* 2004; Iniguez *et al.,* 2004). Bacterial endophytes have been isolated from sterilized plant tissue numerous plant species including both monocotyledons and dicotyledons (Kobayashi and Palumbo, 2000). For isolating facultative endophytes involves the planting of seeds in different soils, allowing the seeds to germinate and the plants to grow, and then macerating surface-sterilized plant tissues and plating the cell sap on bacterial growth medium. The bacterial endophytes belonging to the *Pseudomonas* and *Bacillus* genera are easy to culture, and studies involved culture based isolation and identification of microbes has identified as frequently occurring endophytes (Seghers *et al.,* 2004).

They known to play a crucial role directly; the bacterial endophytes qualify as plant-growth-promoting bacteria (PGPB) when they show ability to colonize and elicit positive effect on the plant growth and development (Hardoim *et al.,* 2008). They are known to promote plant growth indirectly by suppressing the growth of pathogens and eliciting induced systemic resistance (ISR) is well-known in biological control or defense against insect herbivores (Kloepper *et al.,* 1992; Dobbelaere *et al.,* 2003; Van Oosten *et al.,* 2008). Endophytic bacteria have been studied as possible inoculants for increasing plant productivity (Hallmann *et al.,* 1997). The endophytic bacteria have also been reported for their antagonistic activities against phytopathogens, production of siderophores, phytohormones and increased uptake/availability of plant nutrients (Sturz *et al*., 2000; Sessitsch *et al*., 2002). The novel secondary metabolites produced by the endophytic bacteria have been shown to confer better ecological fitness to host plants (Ryu *et al*., 2003; Pirttila *et al.*, 2005).

2.4 Isolation of endophytes from host plants

An important prerequisite for the isolation of endophytic bacteria is the selection of a suitable host. The protocol used for the isolation of endophytes must be appropriate to obtain endophytes present in deep/internal tissues. Further it is imperative to remove all surface contaminants and ensure that the isolation represents only true endophytes without being

contaminated by other microorganisms. Therefore, an isolation methodology for isolation of endophytes plays a crucial role in obtaining the true endophytes.

Attempts have been made, by several researchers to obtain endophytes in pure form from different crops using culture based techniques. Hallmann *et al.,* (1997) reviewed various methods adopted for isolation of endophytes. The most commonly used method was to isolate them from surface-sterilized plant tissues and parts. The entire process of surface sterilization depends on the plant material selected and used for isolation and the possible contaminants available on the surface of plant tissues and suggested that the washing of plant tissue/ tissues in the running tap water and grading of plant material on the basis of their size and surface appearance etc. before used for isolation. In addition, it was recommended to exclusion of any damaged material before surface-sterilization. However, the underground plant parts namely root tissues and rhizomes; thoroughly washing with distilled water was recommended. Afterward neatly cleaned materials were surface sterilized using sodium hypochlorite and 70% ethanol. Following these approaches a number of gram positive and negative bacterial endophytes have been isolated from sterilized plant tissues of several plant species (Puri *et al*., 2005a and b).

Germida *et al.,* (1998) reported that the endophytes population was not so diverse than the root surface population and the endophytes appeared to originate from the latter. Further, several other groups isolated bacterial endophytes from different varieties of Indian beans and other plants (Suman *et al.,* 2001; Araújo *et al.,* 2002; Dent *et al.,* 2004; Zinniel *et al.,* 2002; Sessitsch *et al.*, 2002). Zinniel *et al.,* (2002) isolated more than 800 endophytic isolates from aerial tissues of 4 agronomic crop and 27 prairie plant species. Out of which majority of the isolates were from sorghum and corn; In conclusion, the lesser number of isolates were recovered from perennial plants than from the agronomic crops.

Similarly, Omarjee *et al.,* (2004) isolated endophytic bacteria from the stalks of sugarcane. Among them *Burkholderia* species is an opportunistic endophyte capable of utilizing organic compounds within sugarcane for its growth and survival. It can withstand the physiological conditions encountered within the plants tissues, suppress pathogen growth and avoid induction of host defense responses that could eliminate them Bhore and Sathisha; (2010) isolated 150 potential culturable endophytic bacterial strains, isolated from leaf samples of different plant species collected from Peninsular Malaysia. Most of leaf samples produced 1 or 2 putative endophytic isolates and some had even produced 3 or 4 types of endophytes.

Furthermore, Magnani *et al.,* (2010) isolated several endophytic bacterial colonies from Brazilian sugarcane, majority of bacterial isolates from isolated from stem and leaf tissues of sugarcane were belonging to the family of *Pseudomonaceae* and *Enterobacteriaceae*, respectively. Hung *et al.,* (2007) characterized a total of 32 endophytic bacteria from surface-sterilized root, root nodules and stem tissues of wild and cultivated varieties of soybean. It was noticed that the genetic variability was more among the endophytes isolated from *Glycine max* tissues than from the *G. soja*. Furthermore, Dubey *et al.,* (2010) isolated root nodule endophytic rhizobia from Arhar (*Cajanus cajan*) and identified as *Ensifersino rhizobium* based on their biochemical and physiological characteristics. The *Ensifersino* isolates KCC2 and KCC5 produced siderophore and showed strong antagonistic activities against *Fusarium udum.* Endophytes from roots and root nodules of field pea and chickpea cultivated in Northern India were isolated. Narula *et al.,* (2013) isolated a total of 75 endophytic bacteria roots and nodules of field pea and 88 from roots and root nodules of chickpea demonstrated that presence of gram positive bacteria in the root and root nodules were 50% and 93.4%, respectively and most of the isolates were spore formers (Saini *et al.,* 2013). It was also noticed that the higher number of bacteria were recovered from nodules as compared to the roots of field pea as well as chickpea.

2.5 Morphological and colonization of endophytes

Endophytes are the microbes (bacteria or fungi) that colonize the internal plant tissues without damaging the host or causing any of disease symptoms. Endophytic bacteria could be identified through differential staining; their morphological, physiological and biochemical properties as reported in the *Bergey's Manual of Systematic Bacteriology* (Holt *et al*., 1994).Gram staining techniques widely performed as per modified crystal violet staining method followed by viewing of slides with the light microscope under oil-immersion by (Bathlomew, 1962). Luna *et al.,* (2010) studied the bacterium's natural association with wheat and sorghum after inoculation of seed with bacterium *Gluconacetobacter diazotrophicus* using colony counting and microscopic observation of plant tissues.

2.6 Biochemical characterization of bacterial endophytes

The bacterial endophytes were characterized using the biochemical characteristics. Each microorganism has their own biochemical properties which can be deployed for the putative identification of bacterial genera. These biochemical properties controlled by the enzymatic activities within the cells and they are responsible for bioenergetics, biosynthesis and

biodegradation. These biochemical traits includes, determination of kind of nutrients use of bacteria, the products of its metabolism, the response to specific chemicals and the presence of particular enzymes. Morphological and biochemical characteristics of the isolated endophytes were used to identify the isolated endophytes up-to genus level in various reports. Characterization based on the morphological and biochemical features of bacteria is the most practical way of identifying bacteria to genus and certain species of bacteria. The activities of bacteria namely oxidase, catalase, nitrate reductase, urease, MR and VP, Motility test, carbohydrate utilization test and antibiotic sensitivity tests were widely used for the identification and characterization of bacterial isolates.

2.7 Molecular characterization of bacterial endophytes

Bacterial endophytic communities was characterized using molecular approaches *viz.,* fingerprinting of 16S rRNA and nitrogen fixation (nif H) genes profiling by Polymerase chain reaction (PCR) and visualized with denaturing gradient gel electrophoresis. Studies have been reported for identification and characterization of bacterial endophytes in different crops. However, Hollis *et al.,* (1981) divided/ aligned *Bradyrhizobia japonicum* into three DNA homology groups, one group includes *B. elkanii* and another group *B. liaoningense* comprising extra slow growing *Glycine* isolates, retaining the name *B. japonicum* of *G. max*. High similarity of 16S rDNA gene sequences of bradyrhizobia is the major factor complicating the evaluation of the taxonomic status and interrelationships studies of bradyrhizobia. Several isolates/ strains have 16S rDNA sequence divergences of only 0.1–2.0%. Endophytes from the legume crops have been identified and characterized using various culture dependent and independent techniques. Palaniappan *et al.,* (2010) isolated 39 endophytic bacterial isolates from the nodule of *Lespedeza sp.* grown in South Korea and strains were identified by using 16S rRNA as *Burkholderia, Bradyrhizobium, Bacillus, Arthrobacter, Dyella, Rhizobium, Microbacterium* and *Staphylococcus and Methylobacterium*.

In our country, most of the research work conducted in legume crops related to Plant growth promoting bacteria (PGPR) has been isolated from root and root nodule associated rhizobacteria. There are studies which reported the occurrence of endophytic bacteria from roots and nodules of legumes namely chickpea, bean, alfalfa, cowpea, clover, peanut, pea, soyabean, *fenugreek, Argyrolobium, Acacia, Conzattia,* Lotus, mungbean, *Hedysarum, Leucaena, Kennedia,* Mimosa,

Leucaena, Mimosa, Lotus, mungbean, *Medicago,* Sesbania, Vicia (Dudeja *et al.,* 2012; Dudeja and Nidhi; 2014).

Patel *et al.,* (2012) characterized some endophytes from root and stem tissues of Tomato plants collected from different regions of Gujarat state. A total of 18 bacterial isolates were selected of which one bacterium was identified as *Pseudomonas aeruginosa* by molecular analysis.

Wang *et al.,* (2013) isolated endophytic bacteria associated with root of peanut plants grown in 6 provinces in China using 16S rDNA sequencing. Phylogenetic analysis showed that 49 isolates were belonging to *Bacilli* and 23 isolates to proteo bacteria. It was also reported that the population density of bacterial endophytes found to be highly variable, depending mainly on the host genotypes, developmental stages of the host, density of inoculum and environmental factors/conditions.

Kumar *et al.,* (2013) isolated bacterial endophytes from roots and root-nodules of legumes and roots of non-legumes crops namely, chickpea, field pea, and Lucerne and non-legumes wheat and oat. Reported higher numbers of Gram positive bacteria were present in legume nodules than in its roots and reported the presence of 7 bacterial strains in legume roots and 6 bacterial strains in non-legume roots. About 47.8% bacterial endophytes from legume roots and 56 % from roots samples were solubilizing phosphate (P). Based on sequencing of 16S rRNA gene sequencing *Bacillus licheniformis* strain CRE 1, *Bacillus subtilis* strain CNE 215, and *Bacillus flexus* were identified from chickpea root, root nodule and wheat root, respectively.

Further, Sarma *et al.,* (2014) revealed that study of microbial diversity by partial 16S rRNA gene sequencing analysis of 85 distantly related isolates of fluorescent pseudomonads. Rangjaroen *et al.,* (2014) reported that the endophytic community richness, diversity, consistency and constancy were varied depending on the pigeon pea cultivars, plant tissues, growth and developmental stages of the plants. Further, the community structures of endophytic bacteria across the pigeon pea showed the higher stability than those of the endophytic diazotrophic bacteria. These diversity/ differences in the occurrence of bacterial communities might be influenced by genetic variation in the pigeon pea; however the endophytic diazotrophic bacterial community is affected by the used nitrogen input.

2.8 Mechanisms of endophytic bacteria – host interaction

Plant scan be considered as complex and diverse ecosystems, where different niches are inhabited by vast diversity of bacteria. The interaction of microorganism with its host was influenced by host physiology. The interaction ranged from being positive (mutualism), negative (parasitism/pathogen) to neutral (commensalism). However the endophytic microorganisms obtain their nutrition from the host, in turn they provide enhanced fitness to host plant by increasing the availability of mineral nutrients and also by producing various defense compounds (Sturz *et al.,* 2000; Sessitsch *et al.,* 2002). Additionally a large number of positive impact on plant growth have been accredited to endophytic microbes and include osmotic adjustment, stomata regulation, alteration of root morphology, enhanced uptake/availability of minerals and adjustment of nitrogen accumulation and metabolism (Compant *et al.,* 2005). The microbial cells that complete their one phase of life cycle within the internal plant tissues without causing any apparent symptoms in the host plant are considered as microbial endophytes (Rangjaroen *et al.,* 2014).

Microorganisms associated with plant plays an important role in cycling of nutrients and avoiding/preventing negative effect of phytopathogens. An antimicrobial compound plays a major and crucial role in the suppression of plant pathogens growth by antagonistic effects, especially in the soil/rhizosphere (Raaijmakers *et al.,* 2002). Phytopathogenic fungi and bacteria developed a number of strategies to resist or tolerate the deleterious effects of antimicrobial compound. These mechanisms involve degradation of antimicrobial compounds, active efflux, and interference in the biosynthesis of antimicrobial metabolites etc. (Duffy *et al.,* 2003). The biocontrol activity based on secretion of molecules that kill or reduce the growth of the target pathogen. The bacterial endophytes are known to produce secondary metabolites antibiotics, lipopeptides as an antimicrobial substance.

2.9 Plant growth promotional (PGP) traits of endophytic bacteria

Investigation has been reported on the plant growth-promoting capabilities of numerous endophytes. They enhance the plant growth through the improved cycling of nutrients and minerals such as phosphate, nitrogen and other nutrients. These include phosphate solubilization activity (Verma *et al.,* 2001).

2.9.1 IAA production

Plant hormones are regulators that influence plant growth and development and IAA is one of the most physiologically active auxin. Indole acetic acid (IAA) is a plant hormone with no known apparent function in bacterial cell and it is reported that the production of IAA improve the fitness of the plant microbe/bacterium interaction. Production of IAA allows the bacteria with competitive advantages to colonize the internal plant tissues. Bacterial IAA loosens plant cell wall results an increasing amount of root exudation which provides additional nutrients to support the growth of rhizospheric bacteria (Etesami *et al.*, 2014). Auxins are known to be involved in several plant processes including lateral and adventitious root formation and root elongation, which elevates stress resulting in plant growth promotion (Blanco and Lugtenberg; 2014).

Bacterial IAA can obviate the function of ACC deaminase, siderophore and phosphate solubilizing bacteria. Screening of the endophytic bacteria and rhizosphere for *in-vitro* potential of IAA production provide a reliable basis for identification of effective PGP bacteria. Bacterial auxin can alter the auxin balance inside the plant and is capable of influencing the auxin homeostasis by interfering with plant auxin transport. Bacterial IAA increases root surface area and length and led to provide the plant greater access to soil nutrients and water uptake. Harish *et al.,* (2005) reported that the application of endophytic bacterial strains significantly increased the growth parameters *viz*., pseudo stem height, girth, number of leaves and physiological parameters *viz*., stomatal resistance, chlorophyll stability index and transpiration in legume crops plants both under field conditions and greenhouse conditions.

Bai *et al.,* (2002) reported that the 3 of 14 endophytes improved plant weight and nodulation in soybean when co-inoculated with *B. japonicum.* Blanco and Lugtenberg; (2014) reported that the endophytic bacteria *Rahnella aquaqtilis* and *P. putida* with IAA producing ability that stimulate the growth of radish and some cereals.

Loaces *et al.,* (2011) also stated that the ability to produce IAA was extremely variable among the endophytic bacteria, even with similar ARDRA pattern suggested that the production of IAA is more or less strain dependent.

Endophytes synthesize IAA to direct their plant hosts through signaling pathways. Gaiero *et al*., (2013) reported that the endophytic bacteria *Pseudomonas syringe* can induce IAA and abscisic acid biosynthesis in *Arabidopsis thaliana.*

Bacterial indole acetic acid contributes to circumvent the host defense by regulating/ derepressing the auxin signaling in the plant. IAA production also reported to have direct effect on bacterial survival and its resistance to plant defense (Spaepen *et al.*, 2000).

2.9.2 Phosphate solubilization

Phosphorus is an essential macro-nutrient for required for the growth and development of plants it is mainly involved in several metabolic pathways namely biological oxidation, nutrient uptake and cell division, photosynthesis. Phosphorous deficiency results in the leaves turning brown accompanied by weak stem, small leaves and slow plant development. Plants may take up phosphorus in several forms but the maximum portion of the applied P fertilizer is absorbed in the forms of HPO_4- or H_2PO_4- (Beever and Burns, 2000). Microorganisms are effective and capable of releasing P from inorganic and organic pools of total soil P through the process of solubilization and mineralization.

Phosphate solubilization by Phosphate solubilization bacteria (PSB) strains is associated with the release of low molecular weight organic acids mainly gluconic and ketogluconic (Kim *et al.*, 1997), which through their carboxyl and hydroxyl groups chelate the cations bound to phosphate, therefore converting it into soluble forms. The production of gluconic acid also reported to be the most frequent agent of mineral phosphate solubilization (Shahab and Ahmed; 2008). The ability of PSB has direct correlation with pH of the medium for phosphate solubilization.

Bhatia *et al.,* (2008) reported that the *Pseudomonas* PS1 and PS2 for producing siderophores, indole acetic acid, hydrocyanic acid, and phosphate solubilization under normal growth conditions.

Among the bacterial communities, ecto-rhizospheric strains from *Pseudomonas, Bacilli* and endosymbiotic rhizobia have been described as effective phosphate solubilizers. Bacterial genera namely *Bacillus, Pseudomonas, Rhizobium* and *Enterobacter* along with are the most powerful P solubilizers. *Bacillus circulans, B. megaterium, B. polymyxa, B. sircalmous, B. subtilis, Pseudomonas striata and Enterobacter* is referred as the most important strains (Mohammadi, 2012).

Souza *et al.,* (2013) reported that the 101 isolates were able to solubilize phosphate which were belongs to the genera *Burkholderia, Cronobacter, Pantoea, Enterobacter and Pseudomonas* according to Souza *et al.,* (2013). Plant growth promoting rhizobacteria are

supposed to increase root length and growth resulting in greater root surface area which enables the plant to access more nutrients from soil. In addition, the microorganisms involved in P solubilization as well as can enhance plant growth by enhancing the availability of other trace elements like iron, zinc etc. (Mohammadi *et al*., 2012).

The application of Phosphate solubilization bacteria (PSB) strains in agricultural practice would not only offset the high cost of phosphate fertilizers but would also mobilize insoluble phosphorus available in the fertilizers and soils to which they are applied.

2.9.3 Siderophore production

Siderophores are small and chelating compounds released by microbes to scavenge iron from mineral phases by the formation of soluble Fe 3+ complexes which are takenup through active transport mechanism.

Iron (fe) is a major element desired by several microbes for their cellular processes like electron transport chain and as a cofactor for many enzymes reactions/processes. Microorganisms growing in aerobic conditions need iron for reduction of oxygen hence ATP synthesis, heme formation and for other metabolic activities. In aerobic conditions, the solubility of iron is very low due to the predominance of ferric ($Fe^{3+)}$ ions (Souza *et al.*, 2015).

Production and utilization of siderophore by rhizobacteria is an important aspect because of iron is essential in the nitrogen fixation and assimilation process. Siderophore produced by *P. pseudoalcaligenes* may facilitate the solubility of nutrients such as Fe and other metal and utilization of microbial siderophore by plant and improve the micronutrient availability (Gamit and Tank, 2014). Endophytes compete with plant cells for supply of iron therefore, siderophore production may be highly crucial for endophytic growth. Upreti and Thomas (2015) isolated bacterial endophyte from root tissue of wilt resistant and susceptible tomato cultivars. Microbes showed potential to produce siderophore, HCN and antibiotic biosynthesis. Sansanwal *et al.*, (2018) isolated total 41 endophytes from mungbean only twelve cultures were found positive for siderophore production and 12 bacterial isolates showed HCN production.

2.9.4 HCN production

HCN is known to inhibit the electron transport chain led to disrupting the energy supply to the cells, eventually leading to the pathogen death (Kumar *et al.*, 2013). Among the various biocontrol agents, fluorescent pseudomonads with multiple mode of action for biocontrol of phytopathogens are being used widely as produce a wide variety of growth promoting hormones,

siderophores, antibiotics, HCN and phosphorous solubilization (Saharan and Nehra; 2011). Several environmental factors influenced HCN production by *Pseudomonas sp* and strongly affected by amino acid composition of the culture media. Glycine and proline are the precursor of microbial HCN production.

In rice rhizosphere dominant population of *P. fluorescens, P. aeruginosa and P. putida*was reported by Rangarajan *et al*., (2002). The other bacteria are also associated with root nodules. *Bradyrhizobium*is an important member of PGPR which shows several plant growth promoting activities. These bacteria carry out nitrogen fixation and provide several direct and indirect effects such as phytohormone production, iron-chelation, phosphorous solubilization, hormone production, HCN production, chitinase production, etc. (Deshwal *et al.,* 2003).

Gupta *et al*., (2000) have reported the production of siderophores by *Bradyrhizobium* sp. in *Vigna*. Recently Upreti and Thomas; (2015) isolated bacterial endophyte from root tissue of wilt resistant tomato cultivar Arka Abha and susceptible cultivar Arka. Microbes showed potential to produce HCN and antibiotic biosynthesis.

Sansanwal *et al.,* (2018) isolated total 41 endophytes from mungbean and 12 bacterial isolates showed HCN production.

2.10 Antagonistic effect of endophytes against pathogenic microbes

The plant microbiome is one of the major key determinants of plant health and productivity with deeper understanding of the fundamental, interactions between plants and associated microbes is expected to lead to novel idea for improving plant health. Microbes, which live inter and intra-cellularly in plants without inducing pathogenic symptoms are called endophytes. Endophytes play an important role in the functioning of plants by influencing their development and physiology ranging from providing resistance to biotic and abiotic stress, synthesizing phytohormones, fixing atmospheric nitrogen, biocontrol agents and reciprocal interactions between above and below ground communities.

The application of endophytes as biocontrol agent have emerged as eco-friendly and complementary approach. However, despite the importance of the endophyte- plant relationship, knowledge on the association of bacterial endophytes and their interactions with host- pathogen under hostile environmental conditions in Pigeon pea still limited. Entophytic bacteria are known to minimize or prevent the deleterious effects of pathogenic organisms. The diseases caused by the bacterial, fungal, viral origin and in some instances even damage caused by nematodes and

insects can be reduced following prior inoculation with endophytes (Sturz *et al.*, 2000; Berg and Hallmann; 2006).

Upreti and Thomas; (2015) isolated bacterial endophyte from root tissue of wilt (*Ralstonia solanacearum*) resistant tomato cultivar Arka Abha and susceptible cv. Arka. Microbes screened for general indicators of biocontrol potential for siderophore, HCN and antibiotic biosynthesis and internal colonization of roots by endophytes was confirmed through confocal imaging after SYTO-9 staining.

Abdallah *et al.,* (2016) conducted experiment on biocontrol of Fusarium wilt and plant growth promotion of tomato using bacterial endophytes isolated from *Nicotiana glauca* organs. The study reported that the *Nicotiana glauca* isolates were the most effective in decreasing yellowing and wilt symptoms by 94 and 88% and the vascular browning extent by 95–97.5%, respectively, as compared to FOL-inoculated and untreated control.

Comby *et al.,* (2017) isolated 86 endophytic bacteria from inner tissues of wheat plants and screened for inhibition of growth of *Fusarium graminearum* by *in vitro* dual culture assays. A total of 22 strains appeared very effective to inhibit *Fusarium graminearum* (inhibition of 30–51%). Bahroun *et al.,* (2018) isolated bacterial endophytes from nodule of *Vicia faba* and *Cicer aeriteum* and screened *in vitro* test against *Fusarium solani* and potential isolates having biocontrol activities tested *in planta* and found three strains showing effective control on fusarium root rot.

CHAPTER-III
MATERIALS AND METHODS

The present study entitled on "Isolation and characterization of endophytic microbes from Pigeon pea (*Cajanus cajan* L)" was carried out at ICAR - National Institute of Biotic Stress Management (NIBSM), Baronda, Raipur. The materials and methods used during course of the present investigation are described below:

3.1 Materials

3.1.1 Plant materials

Pigeon pea (*Cajanus cajan* L) varieties namely Asha, UPAS 120, BDN-2, Rajeevlochan, Laxmi, Rajeshwari, Raipur local were used for the study.

3.1.2 Chemicals, buffers & reagents

All the chemicals/reagents used for the study were of molecular and analytical grades. They were procured from the standard manufacturers *viz;* Qiagen, Applied Biosystems, Fermentas, Sigma, Invitrogen, Amaresco, Thermo Scientific, Himedia and Merck. The reagents composition, components different buffers & media used are given in Appendix I.

3.1.3 Glassware and plasticware

All glassware used for conducting the experiments under present investigation were of Borosil makes. The glasswares were rinsed with distilled water and were kept in an oven at 250°C overnight before use. The plasticware items like micropipette tips, PCR and eppendorf tubes were of used for DNA isolation, PCR and sequencing were congruent for molecular biology grade.

3.1.4 Equipments used in the experiments

S. No.	Generic name of the Equipments	Make/Manufacturers
1.	Micro-centrifuge	Eppendorf
2.	PCR machine	Eppendorf, Mastercycler pro S
3.	Spectrophotometer	Eppendorf
4.	Gel electrophoresis system	CBS Scientific
5.	Gel documentation system	Protein simple
6.	Incubator shaker	Eppendorf , New Brunswick

S. No.	Generic name of the Equipments	Make/Manufacturers
7.	-80°C Deep freezer	Eppendorf
8.	-20°C Deep freezer	Thermo Scientific
9.	Electronic Balance	Sartorius
10.	BOD incubator	Eppendorf
11.	Laminar Air Flow	IMSET
12.	Micro-Oven	AFB
13.	Water Bath	Labtech
14.	UV Trans-illuminator	Protein simple

3.2 Methods

3.2.1 Isolation of endophytic bacteria from pigeon pea

3.2.1.1 Preparation of media

The nutrient agar medium was used for the isolation of endophytic bacteria (Appendix I). The medium was sterilized by autoclaving and cooled to room temperature. Solidified media was prepared by the addition of 1.5g/100ml agar before autoclaving and pouring on plates under laminar air flow in aseptic condition.

3.2.1.2 Collection of samples

Leaf, stem, root, pods and flower were collected from pigeon pea (*Cajanus cajan*) varieties namely Asha, UPAS 120, BDN-2, Rajeevlochan, Laxmi, Rajeshwari, Raipur local plants grown in the pots and research fields at ICAR-National Biotic Stress Management, (Baronda) Raipur. Healthy plants were carefully removed, washed under tap water to remove soil. Different tissues/ parts samples were collected in separate beaker, soaked in distilled water and drained.

3.2.1.3 Sterilization of samples

For surface sterilization, leaf, stem and root samples were treated with 3% $HgCl_2$ for 3 minutes while flower and pod samples were treated with 1% $HgCl_2$. The samples were then sterilized with 70% ethanol for 5 min. After sterilization respective tissues were washed thrice with sterile distilled water and dried in laminar air flow. Surface disinfestations parameters like

selection of disinfectant, its strength, duration of immersion in disinfectant were optimized prior to experimentation.

3.2.1.4 Culturing of samples on culture media

The cut ends of surface sterilized segments were removed with flame sterilized scalpel and were placed in nutrient agar plate with the cut surface touching the agar. The plates were incubated for two to eight days at 35±2°C in a B.O.D incubator. To check sterility, the shoots were rolled on nutrient agar plates as well as 0.1 ml aliquot from the final wash was inoculated to nutrient agar plates. Samples were discarded if any growth was detected in the sterility check. Surface disinfected tissues were aseptically placed on different medium, for the isolation of specific bacteria. Sterility check was performed by plating the last wash on same media. Plates were incubated at 35±2°C for 2-5 days and observed daily. Growth of bacteria around the inoculated samples were carefully observed, isolated and purified by streaking over the surface of their specific media *viz*. nutrient agar to obtain a single distinct colony.

3.2.1.5 Selection of endophytic bacterial isolates

The endophytic bacterial isolates were screened based on their morphological characters and growth in NA media. All selected isolates were then purified by streaking on NA plates. Glycerol stock was prepared to store the cultures for a longer time at refrigerated conditions (-80°C). Short-term storage for further characterization was on nutrient agar plates at 4°C.

3.2.2 Characterization of the endophytic isolates

3.2.2.1 Morphological characterization and colonization study of bacterial endophytes

Bacterial isolates from the different plant parts were purified to obtain single colony. The pure culture was observed for morphological characteristics on the basis of colony color, shape, texture, elevation and size.

3.2.2.2 Gram staining and microscopic observation

Gram reaction and motility were carried out to characterize the tentatively identified endophytes. Gram staining was carried out as per modified Crystal violet gram staining method. The slides were viewed with the light microscope under oil-immersion.

3.2.2.3 Potassium hydroxide test (KOH)

One drop of 3% KOH was taken on a clean microscopic slide and a loop was used to transfer a generous amount of bacteria (cultured for 24-48 h) to the drop of KOH carefully and

mixed properly. After mixing, the loop was elevated to observe for the formation of mucoid string within 30 sec indicating the gram negative bacteria.

3.2.2.4 Growth on MacConkey agar (MCA)

MacConkey Agar medium (Appendix I) is both selective and differential, which inhibit the growth of gram positive bacteria and supports the growth of gram-negative bacteria particularly *Enterobactariaceae* family on this MCA plate. Gram negative bacterial isolates were streaked in MCA plate and incubated in 37°C temperature and observed for 2 days. The differential ingredient present in the media is lactose and bile salts. Fermentation of the sugar results in an acidic pH and causes the pH indicator, neutral red, to turn a bright pinky-red color. The organisms causing acid production with development of pink color colonies are lactose fermenter bacteria while those with pale colonies due to alkaline reaction are non lactose fermenter bacteria.

3.2.3 Biochemical characterization of bacterial endophytes

3.2.3.1 Catalase activity

A loopful of overnight grown culture of endophytic bacterial isolates maintained on nutrient agar plates were transferred to a glass slides and mixed thoroughly with 2-3 drops of 3 % H_2O_2 solution and observed for the presence of the effervescence (production of gas bubbles). Production of gas bubbles immediately indicates the presence of catalase positive reaction otherwise catalase negative.

3.2.3.2 Oxidase activity

The endophytic bacterial culture were streaked on oxidase disc after streaking within 7-10 second if the disc are given purple colour in streak side these are oxidase positive otherwise result are negative.

3.2.3.3 Nitrate reduction test

The isolates were inoculated into 10 ml of sterile nitrate broth in test tubes and incubated at 37°C. After 24 hours, culture broth was tested by adding equal amounts (3-4 drops) of sulfanilic acid and alpha naphthylamine. Development of red color indicated the positive result in which nitrate had been reduced to nitrite. The nitrate broth tubes in which red color didn’t appear were added with pinch of zinc dust. The tubes in which the color changes to red after addition of zinc dust were considered as negative while the tube without any change in colour were considered as positive as the such tubes the nitrate has been reduced to nitrogen gas.

3.2.3.4 Methyl red and Voges-Proskauer (MR-VP) test

The Methyl Red, Voges-Proskauer (MR-VP) broth prepared in two sets were inoculated with the selected bacterial isolates and incubated at 30°C for 48 hrs. To the first set of tubes, few drops of an alcoholic solution of methyl red indicator were added. The development of distinct red color was indicative of positive reaction for MR test in which glucose was converted into acid and gas by the endophytic bacteria. For Voges-Proskauer test, to the second set 3-4 drops of Barritt A and then Barritt B was added, shaken gently for 1-2 min and kept at room temperature for 30 minutes. The positive reaction of acetyl methyl carbinol production was indicated by development of red color. This indicates positive result for the VP test.

3.2.3.5 Citrate utilization test

Endophytic isolates were inoculated into test tubes having Simmons citrate agar medium and incubated for 48 h at 30°C. Simmons citrate agar contained citrate as its sole carbon and energy source. The presence of growth and change of color from green to blue due to pH change indicated positive reaction.

3.2.3.6 Motility indole urea agar (MIU)

The sterile Motility Indole urea Agar (MIU) tubes having 5 ml medium were prepared and stab inoculated with isolated endophyte cultures and incubated at 37°C. After 24-48hrs, were observed for turbidity. If the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark and are motile. While if the stab mark is clearly visible and the rest of the tube is not turbid, then the organism is considered as non-motile.

3.2.3.7 Triple sugar iron (TSI) assay

The Triple sugar iron (TSI) test is a microbiological test roughly named for its ability to test a microorganism's ability to ferment sugars and to produce hydrogen sulfide gases. It is often used in the selective identification of enteric bacteria including *Salmonella* and *Shigella*.

Firstly, prepared Triple sugar Iron media (TSI) and transfer on test tube in slant position to make butt and slant in tube. The tubes inoculated with pure (thoroughly stab with streak culture) culture of the isolated endophytes. It was incubated at 37°C for 5 days and the tubes were observed daily. Bacteria that ferment any of the three sugars (glucose, lactose and sucrose) in the medium will produce by products. These by products are usually acids, which will change the color of the red pH-sensitive dye (phenol red) to a yellow color. Position of the color change distinguishes the acid production associated with glucose fermentation from the acidic by

products of lactose or sucrose fermentation. Many bacteria that can ferment sugars in the anaerobic butt of the tube are enterobacteria. Some bacteria utilize thiosulfate anion as terminal electron acceptor, reducing it to sulfide. If this occurs, the newly formed (H2S) reacts with ferrous sulfate in the medium form ferrous sulfide which is visible by black precipitate. The blackening of the medium is almost always observed in the butt (bottom) of the medium.

3.2.3.8 Antibiotic sensitivity assay

Antibiotic sensitivity assay was done as per the Bauer-Kirby method. Muller Hinton Agar (MHA) media were prepared and poured into the sterile Petri plates. Bacterial lawn was prepared by spreading 0.1 ml of the exponential phase culture of endophytic isolate (having turbidity of 0.5 McFarland) onto the agar plates with the help of sterile spreader. Aseptically about 14 antibiotic discs from Himedia [namely Ampicillin (AMP) 10 mcg, Streptomycin (S) 10 mcg, Polymyxin (PB) 300 units, Cifixime (CFM) 5 mcg, Vancomycin (VA) 30 mcg, Gatifloxacin (GAT) 5 mcg, Methicillin (MET) 5 mcg, Ciprofloxacin (CIP) 5 mcg , Ceftriaxone (CTR) 30 mcg, Tetracycline (TE) 30 mcg, Nalidixic acid (NA) 30 mcg, Gentamicin (GEN) 30 mcg, Trimethoprim (TR) 5 mcg, Azithromycin (AZM) 15 mcg)] were placed on the surface of bacterial lawn of each bacterial isolates at a distance of approx. 2 cm apart. All the Petri plates were incubated at 35±2°C for 24-48 h and observed by the measuring the zone of complete inhibition around the disc. The zone measured in mm was interpreted as mention in zone size interpretative chart of Himedia and the endophytic culture was recorded as sensitive or resistant to antibiotic on the basis of interpretative criteria of chart.

3.2.4 Molecular characterization of bacterial endophytes

3.2.4.1 Isolation of total genomic DNA from bacterial endophytes

Total genomic DNA from bacterial endophytes was isolated using QIAamp DNA Mini Kit as per the manufacturer's protocol. The brief procedure of DNA isolation process as mentioned below:

- The endophytic bacterial isolates were grown in 5 ml of Nutrient Broth (NB) overnight at 37°C with constant shaking at 150 rpm.
- The 1500 µl bacterial suspension was transferred to 2 ml sterile eppendorf tube and centrifuged at 10000 rpm for 10 min to form pellet.
- Supernatant was discarded and pellet was re-suspended in 180 µl of Extraction buffer AL by

pipetting up and down repeatedly, to this 20 µl of proteinase k was added to precipitate cellular proteins after vortex.

- Samples were incubated in water bath at 65°C for 1-1.30 hrs.
- To this 200 µl ATL buffer was added and incubated in water bath for 10 min.
- 200 µl ethanol was added to the lysate.
- Lysate was transferred in to spin column and centrifuge at 8000 rpm for 1 min. and supernatant was discarded.
- To the spin column 500 µl AW1 buffer was added and centrifuge at 8000 rpm for 1 min. supernatant was discarded
- Spin column was again washed by addition and 500 µl AW2 buffer and centrifuge at 8000 rpm for 1 min. supernatant was discarded
- Spin column was transferred to new 1.5 ml eppendorf tube, 50 µl of elution buffer AE was added and kept for 2 min. and centrifuge at 8000 rpm for 1 min.
- Genomic DNA was eluted and stored at - 20°C deep freezer for further use.

3.2.4.2 Assessment of purity and quantity of DNA

The qualitative and quantitative analysis of isolated genomic DNA was performed using spectrophotometer (Eppendorf). Two microlitre DNA was loaded into the micro well and the concentrations of samples and ratio at $A_{260}/A_{280,}$ A_{260}/A_{230} were measured. The quality of DNA also was checked on 0.8 % (w/v) agarose gel. 5 µl of extracted genomic DNA of each samples mixed with 1 µl of 6X gel loading dye and was loaded in each well and electrophoresis was carried out at 70 V for 30 to 45 minutes. The bands were visualized under UV light using a Transilluminator.

3.2.4.3 PCR amplification of 16S rDNA gene

Identification of the bacterial species was based on the sequencing of the 16S rRNA, using the primer 27 F and 1492 R (Lane, 1991) were used to amplify ~1500 bp region of 16S rRNA gene using a thermal cycler (Eppendorf, Mastercycler ProS, USA).

Primer Name	**Primer sequences (5-3)**
Forward primer 27F	5’-AGTTGATCCTGGCTCAG -3’
Reverse primer 1492R	5’-ACC TTG TTA CGA CTT-3’

The composition of the reaction mixture

S.No.	Components	Value/Quantity
1	*Taq* DNA polymerase buffer (10X)	2.5 µl
2	dNTP mixture	10 mM
3	Forward primer (27F)	10 µm
4	Reverse primer (1492R)	10 µm
5	*Taq* DNA polymerase	2 units
6	Genomic DNA (50 ng)	50-75 ng
	Total volume	25 µl

The reaction mixture was given a short spin in a microcenrifuge for mixing of the cocktail components. The PCR tubes were then loaded in a thermal cycler

Thermal cycling conditions for PCR amplification

Sr. No.	**Steps**	**Temperature (°C)**	**Duration**
1.	Initial Denaturation	94	4 min.
2.	Denaturation	94	45 sec.
3.	Annealing	56	45 sec.
4.	Extension	72	90 sec.
Repeated	35 times to step 2		
5.	Final Extension	72	7 min.
6.	Hold	4	-

3.2.4.4 Gel analysis of PCR product and sequencing

To confirm the targeted PCR amplification, all the PCR products were run on 1.2 % agarose gel containing 0.5 µg/ml ethidium bromide. 10 µl of PCR product was mixed with 2 µL of 6 x gel loading dye and loaded into the well. The 50 bp DNA ladder (Fermentas) was also loaded along with the samples. The electrophoresis was conducted at 100 V to separate the amplified bands. The gel was visualized under UV transilluminator and documented by gel documentation system (Protein simple, Alfa Innotech Corporation, USA). Primer pairs produced amplicon of expected size was used for gel elution.

3.2.4.5 DNA extraction from gel (Gel elution)

The amplicons were gel eluted using Min-elute gel elution kit following manufacturers instructions (Qiagen). Agarose 1.2 g was used to make the gel in 100 ml of 1 X TAE buffer. The solution of agarose and 1 X TAE buffer was heated up to 1 min to dissolve agarose. When cooled a bit, the gel was poured into the gel cast, the comb was fixed and the gel was allowed to solidify for 20 min. Each sample prepared for running on gel had a volume of 40 µl mixed with 3 µl of loading dye. 100 bp ladder was used to ascertain the size of band. The gel was allowed to run for 40 min at 90 V. The bands on gel were visualized and cut under UV transilluminator and quantified at 260 nm using a spectrophotometer (Eppendorf). The purified 16S rDNA amplicons were sequenced in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, CA, and USA).

3.2.4.6 Analysis of 16S rDNA sequences

The sequencing of 16S rDNA was performed using both forward and reverse primers. The generated sequences were aligned using the BioEdit software, V. 7.2.5, and compared with NCBI databases and sequences showing [99 % similarity by Nucleotide Basic Local Alignment Search Tool (BLASTn) program available at the National Center for Biotechnology Information (NCBI) BLAST server (www.ncbi.nlm.nih.gov/ BLAST) (http://www.ncbi.nlm.nih.gov/blast/) (Altschul, 1997). The nucleotide sequences determined in this study have been deposited in the Gene Bank database and accession numbers were obtained.

3.2.5 Plant Growth Promoting (PGP) traits

3.2.5.1 Indole acetic acid (IAA) production

Endophyte isolates were grown in certified tryptone water (10 ml) supplemented with tryptophan (0.01%) for 5 days and the production of IAA was examined daily by processing the 2ml aliquot of medium for detection of indole. Bacterial cultures (2ml) were centrifuged at 8000 rpm for 10 minutes at 4°C to harvest the cells. 0.5 ml supernatant was taken into a fresh microcentrifuge tube and 2 drops ortho phosphoric acid ($FeCl_3$ + H_2SO_4) and 1 ml Salkowaski reagent were added and kept at room temperature for 30 min and observed for the development of color. Pink color in media indicated the presence of IAA and such isolates were selected for further study.

3.2.5.2 Siderophore production

Endophytic bacterial isolates were assayed for qualitative siderophore production on the Chrome azurol succinate (CAS) agar medium described by Schwyn and Neilands (1987). The CAS agar medium plates were spot inoculated with test organism and incubated at 28± 1°C for 2-4 days. Development of yellow–orange halo zone around the spot was considered as positive for siderophore production.

3.2.5.3 Phosphate solubilization

Endophytic bacterial isolates were spot streaked on Pikovskya's agar plates for detection of phosphate solubilization. Plates were incubated at 37±1°C for 72 hrs and observed for formation of clearing zones around the colonies due solubilization of inorganic phosphate by bacterial isolates. The appearance of clear zone around the spot inoculated colonies was considered as positive for phosphatase test.

3.2.5.4 Fluorescent pigment production

Endophytic bacterial isolates were spot inoculated on King's B medium plates and incubated at 28°C for 2 days. Plates were observed under UV light to examine fluorescent ability. Presence of yellowish green fluorescence colour indicated positive for fluorescent pigment production.

3.2.5.5 HCN production

Exponentially grown endophytic bacterial isolate separately streaked on glycerin agar medium as described by Bakker and Schippers, 1987 with simultaneous supplementation of a filter paper soaked in picric acid (0.5%) in Na_2CO_3(5%) in the upper lid of Petri dish. The plates were incubated at 28±2°C for 24 to 48 hours. Change in colour of filter paper from yellow to light brown for moderate (brown) or strong (reddish-brown) indicated HCN production.

3.2.6 Antagonistic activities

3.2.6.1 *In vitro* antagonistic activities against soil borne fungal pathogens

Antagonistic test was conducted on PDA culture media using Dual Plate assay. Soil borne fungal pathogens namely *Sclerotium rolfsii* and *Fusarium species* isolated from Pigeonpea were used to test antagonistic activities of bacterial endophytes isolated from different tissues of Pigeonpea. A 5 mm mycelia mat of each soil-borne fungi, *Sclerotium rolfsii* and *Fusarium species* were placed on one side of a potato dextrose agar (PDA) (HiMedia Laboratories, Mumbai, India) and each endophytic bacterial isolate were streaked on the other side of the

medium. The PDA plate was cultured at 28◦C for 7 to 14 days. During the cultivation, antagonistic effects of the bacterial isolates against the fungal isolates were confirmed by inhibition zones formed between the endophytic bacterial and fungal isolates. The dual culture was performed in three replicates. The antagonistic activities exhibited by bacterial endophytes confirmed twice and the zone of inhibition was recorded.

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation entitled on "Isolation and characterization of endophytic bacteria from pigeon pea (*Cajanus cajan* L.)"was carried at ICAR-National Institute of Biotic Stress Management (NIBSM), Baronda, Raipur. The results are presented in this chapter under the following headings:

4.1 Isolation of endophytic bacteria

The bacterial endophytes were isolated from seven pigeonpea varieties/ lines (PL-1 to PL-7) and five plant tissues namely root, stem, leaf, flower and pod. A total of 45 endophytic bacterial isolates were isolated and pure cultured from the different samples. The number of bacterial isolates isolated from different tissues was19, 11, 11, 2and 2 from the root, stem, leaf, flower and pod samples, respectively. The detail of pigeonpea lines and bacterial endophytes isolated from these lines mentioned in Table 4.1 and Plate 4.1.

Table 1: Detail of samples used for the isolation of bacterial endophytes from pigeon pea.

S.No	Sample code	Name of pigeonpea lines /varieties	Root	Stem	Leaf	Flower	Pod	Grand Total
1.	PL-1	Asha	2	3	1	-	-	6
2.	PL-2	UPAS-120	4	1	-	-	-	5
3.	PL-3	BDN-2	2	2	-	2	2	8
4.	PL-4	Rajeevlochan	2	3	3	-	-	8
5.	PL-5	Laxmi	3	2	-	-	-	5
6.	PL-6	Rajeshwari	2	-	4	-	-	6
7.	PL-7	Raipur local	4	-	3	-	-	7
		Total isolates	**19**	**11**	**11**	**2**	**2**	**45**

4.2Morphological and cultural characteristics of bacterial endophytes

The purified bacterial endophytes isolates were examined for the morphological and colony characteristics like, shape, size, margin, growth pattern etc. On the basis of morphological observations on nutrient agar medium, 16 bacterial isolates produced medium sized, irregular

shaped, off-white and rough colonies. While 18bacterialisolates produced round shaped and raised colonies with entire margins, mucoid and cream in color and 10 isolates produced medium sized, round shaped and flat colonies with smooth margin and light yellow to off white color. Further 3 isolates produced a fluorescent green pigment on King's B medium were grouped in *Pseudomonas spp*. 1 endophytic bacterial isolate produced medium size, round, regular shape, flat, red color identified as *Fictibacillus phosphorivorans*. The size of most of the isolated varied from pin to medium. Most of the bacterial endophytes (36) have individual rod shape and (8) cocco-bacilli and coccii, while (1) bacteria had capsulated form.

The microscopic observation of gram stained slides through compound microscope revealed that out of 45 isolates 28 were gram positive and 17 were gram negative bacteria. Microscopic morphological observation of isolates showed thin, short, thick, chain formation, individual and some bunch formed, capsulated and present endospore. The details of morphological and cultural characteristics of bacterial endophytes are mentioned in Table 4.2 and Plate 4.2 and4.3. The microbes were grown on selective culture media (Plate 4.4) designated for the identification of gram positive and negative isolates and identification of *Bacillus* and *Pseudomonas* isolates.

4.3 Biochemical characterization of endophytic bacterial isolates of pigeon pea.

Gram's staining showed that the out of 45 bacterial endophytes (28) 62.2%were Gram positive and (17) 37. 7% were Gram negative (Table 4.3). Biochemical characterization of endophytic bacterial isolates for different traits showed that the Oxidase (24) 53.2%, Catalase (30) 66.6%, Citrate utilization (21) 46.6%, Urease (10) 22.2%, Nitrate reductase test (18) 40%, Methyl Red (MR) (9) 20%, Vogus Proskauer (VP) (3) 6.6% and motility (22)48.8%, showed positive reaction. Triple sugar iron (TSI) test showed that 10 isolates were able to produce blackening and 6 isolates have ability to produce gas. The detail of biochemical characteristics of bacterial endophytes are mentioned in Table 4.3 and Figure 4.1and Plates 4.5, 4.6, 4.7.

More number of bacteria was present in nodules as compared to the roots of fieldpea as well as pigeonpea. The results are in concurrence with the Dubey *et al.* (2010)isolated 8 strains of endophytic root nodule rhizobia from pigeon pea (*Cajanus cajan*) and characterized based on their physiological and biochemical properties and identified as the isolates as *Ensifer sinorhizobium*.

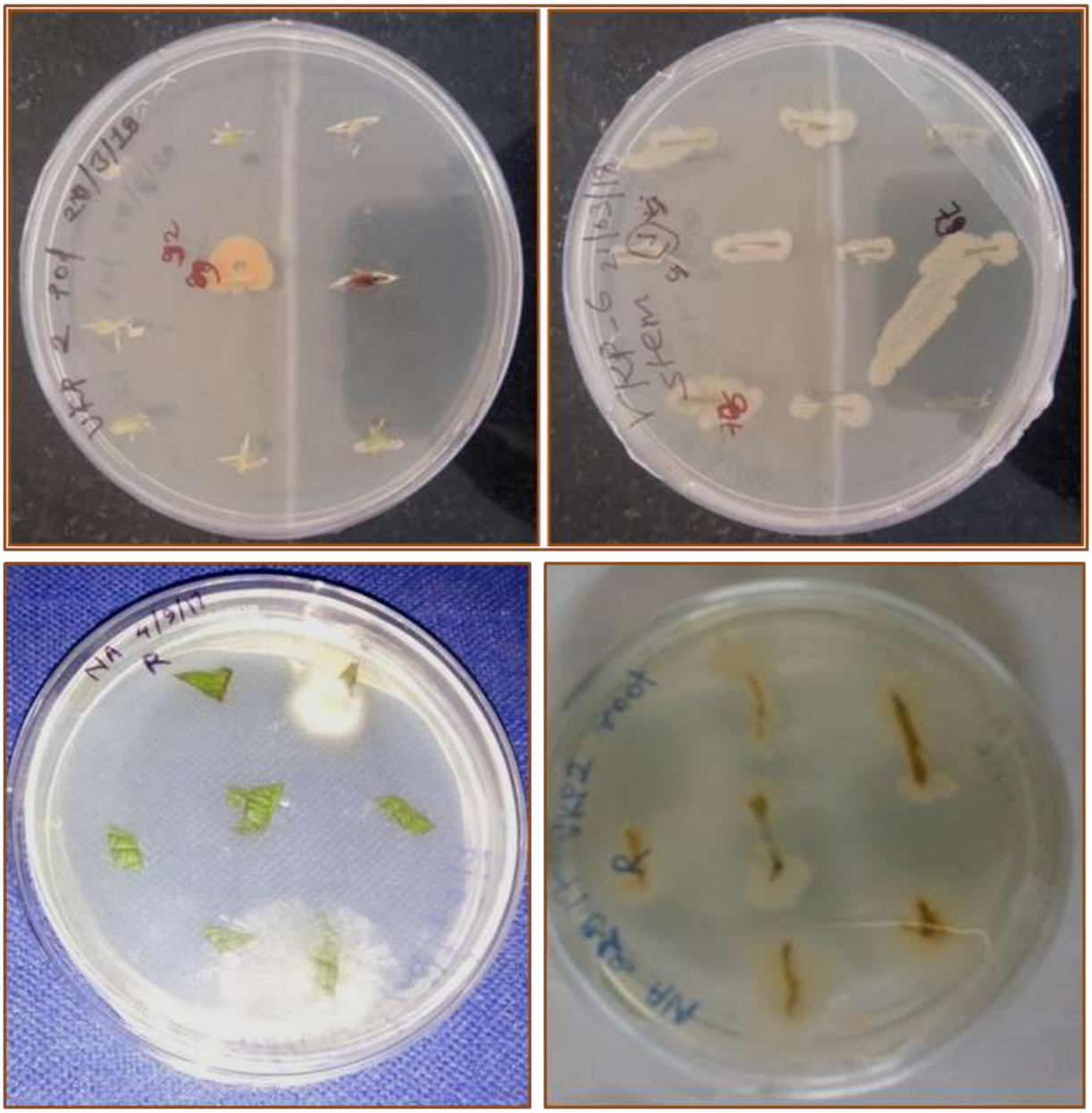


Plate: 4.1 Initiation of bacterial culture and isolation of pure bacterial endophytes

Table 4.2: Morphological and cultural characteristics of bacterial endophytes

S. No.	Name of the bacterial endophytic isolates	Pigeonpea lines/Varieties	Plant tissues	Colony characteristics	Shape of organism
1.	NIBSM_PR1	PL-1	Root	Whitish, dome shape, irregular, spreading type, medium size	Long, individual rod
2.	NIBSM_PR2	PL-1	Root	Whitish, dome shape, irregular, spreading type, medium size	Small rod
3.	NIBSM_PR3	PL-2	Root	Yellowish, dome shape, round, mucoid, pin size	Very short, individual rod
4.	NIBSM_PR4	PL-2	Root	Whitish, round, irregular margin, flat, rough medium size	Rod chain forming, bipolar bodies at the end
5.	NIBSM_PR5	PL-2	Root	light yellow, flat, medium size	Coccobacilli
6.	NIBSM_PR6	PL-2	Root	Dark yellow, dome shape, mucoid, medium size	Thin, long rod
7.	NIBSM_PR7	PL-3	Root	Creamish, dome shape, spreading type, oval, medium size	Rod, bipolar spores chain forming
8.	NIBSM_PR8	PL-3	Root	Light yellowish, dome shape, mucoid type, pin size	Short rod
9.	NIBSM_PR9	PL-4	Root	Whitish, dome shape, medium size	Rod, bipolar bodies, short chain and in bunch
10.	NIBSM_PR10	PL-4	Root	Whitish, round, regular margin, sticky, flat, medium size	Short rod, spore present
11.	NIBSM_PR11	PL-5	Root	Whitish, dome shape, spreading type, rough, medium size	Short rod
12.	NIBSM_PR12	PL-5	Root	Whitish, rough irregular margin, thread like structure	Short rod
13.	NIBSM_PR13	PL-5	Root	Creamish, dome, irregular, spreading type, medium size	Short and thick rod
14.	NIBSM_PR14	PL-6	Root	Light yellow, dome shape, round, mucoid, medium size	Coccobacilli
15.	NIBSM_PR15	PL-6	Root	Creamish, dome, round, medium size	Bacilli long, thick, short chain, square end spore forming

16.	NIBSM_PR16	PL-7	Root	Yellowish, dome shape , round mucoid, pin size	Individual and small rod
17.	NIBSM_PR17	PL-7	Root	Creamish, dome shape, round, mucoid, medium size	Coccobacilli, bunch shape
18.	NIBSM_PR18	PL-7	Root	Whitish, round, regular, smooth, sticky, pin size	Short and thin rod
19.	NIBSM_PR19	PL-7	Root	Dark-red, spreading type, oval, medium size	Individual coccii
20.	NIBSM_PS1	PL-1	Stem	Creamish, irregular margin, spreading type, large size	Individual and also in bunch, small rod, central endospore
21.	NIBSM_PS2	PL-1	Stem	Creamish, flat, round, medium size	Individual rod, thick and long capsulated
22.	NIBSM_PS3	PL-1	Stem	Creamish, flat, round, medium size	Rod shape,
23.	NIBSM_PS4	PL-2	Stem	Creamish, dome shape, irregular, medium size	Short, thin rods
24.	NIBSM_PS5	PL-3	Stem	Creamish ,dome shape, oval, pin size	Coccobacilli
25.	NIBSM_PS6	PL-3	Stem	Creamish, oval, mucoid type, pin size	Very short rod
26.	NIBSM_PS7	PL-4	Stem	Creamish, irregular, oval shape, large size	Coccobacilli
27.	NIBSM_PS8	PL-4	Stem	Yellowish, dome shape, round, medium size	Coccobacilli
28.	NIBSM_PS9	PL-4	Stem	Light yellow, dome shape, pin, mucoid type	Short, thin rods
29.	NIBSM_PS10	PL-5	Stem	Whitish, round, irregular margin, sticky, flat	Thick and long rod in chain spores visible
30.	NIBSM_PS11	PL-5	Stem	Whitish, round shape, irregular, dry, spreading, pin size	Individual, thin, small, short rod
31.	NIBSM_PL1	PL-1	Leaf	Whitish, dome shape, oval, medium size	Individual rod, short, thick, central spore
32.	NIBSM_PL2	PL-4	Leaf	Creamish, dome shape, round, medium size	Rod, central spore forming

33.	NIBSM_PL3	PL-4	Leaf	Whitish, round, dome shape, medium size	Very short rod, spore present
34.	NIBSM_PL4	PL-4	Leaf	Whitish, flat, round, irregular margin, dry, spreading, pin point size	Rod, thick and long, central spore
35.	NIBSM_PL5	PL-6	Leaf	Whitish, round shape, regular, smooth, medium size	Thin, long rod, central endospore
36.	NIBSM_PL6	PL-6	Leaf	Creamish, dome shape, round, pin size	Rod, thick and large number of spores
37.	NIBSM_PL7	PL-6	Leaf	Creamish, dome shape, round, pin size	Thick, long rod in chain, central endospore
38.	NIBSM_PL8	PL-6	Leaf	Creamish, round shape, flat, medium size	Long rod, thick, capsulated
39.	NIBSM_PL9	PL-7	Leaf	Light creamish, oval, medium size	Individual and small rod
40.	NIBSM_PL10	PL-7	Leaf	Light yellowish, dome, mucoid, pin size	Individual Coccobacilli
41.	NIBSM_PL11	PL-7	Leaf	Light yellowish, dome shape, round, pin size	Coccobacilli
42.	NIBSM_PF1	PL-3	Flower	Whitish, round shape, regular, flat, smooth, pin size	Individual rod, thick. small, central endospore
43.	NIBSM_PF2	PL-3	Flower	Creamish, dome shape, round, medium size	Individual rod, long and thick
44.	NIBSM_PP1	PL-3	Pod	Light creamish, round shape, flat, pin size	Small rod in chain form
45.	NIBSM_PP2	PL-3	Pod	Whitish, round shape, irregular, smooth sticky, pin size	Small rod in individual form

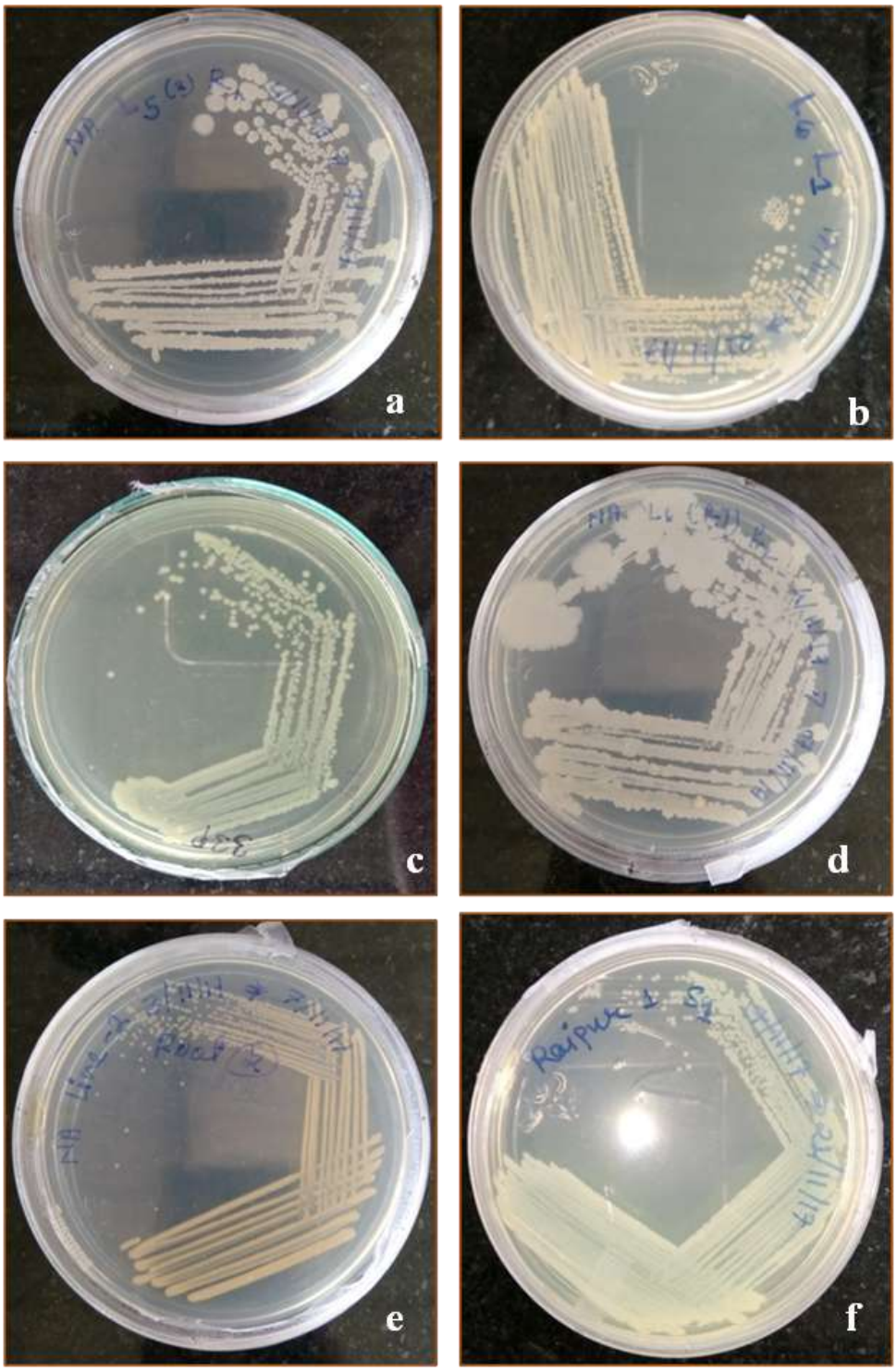


Plate: 4.2 Isolation of bacterial endophytes and their colony characterization

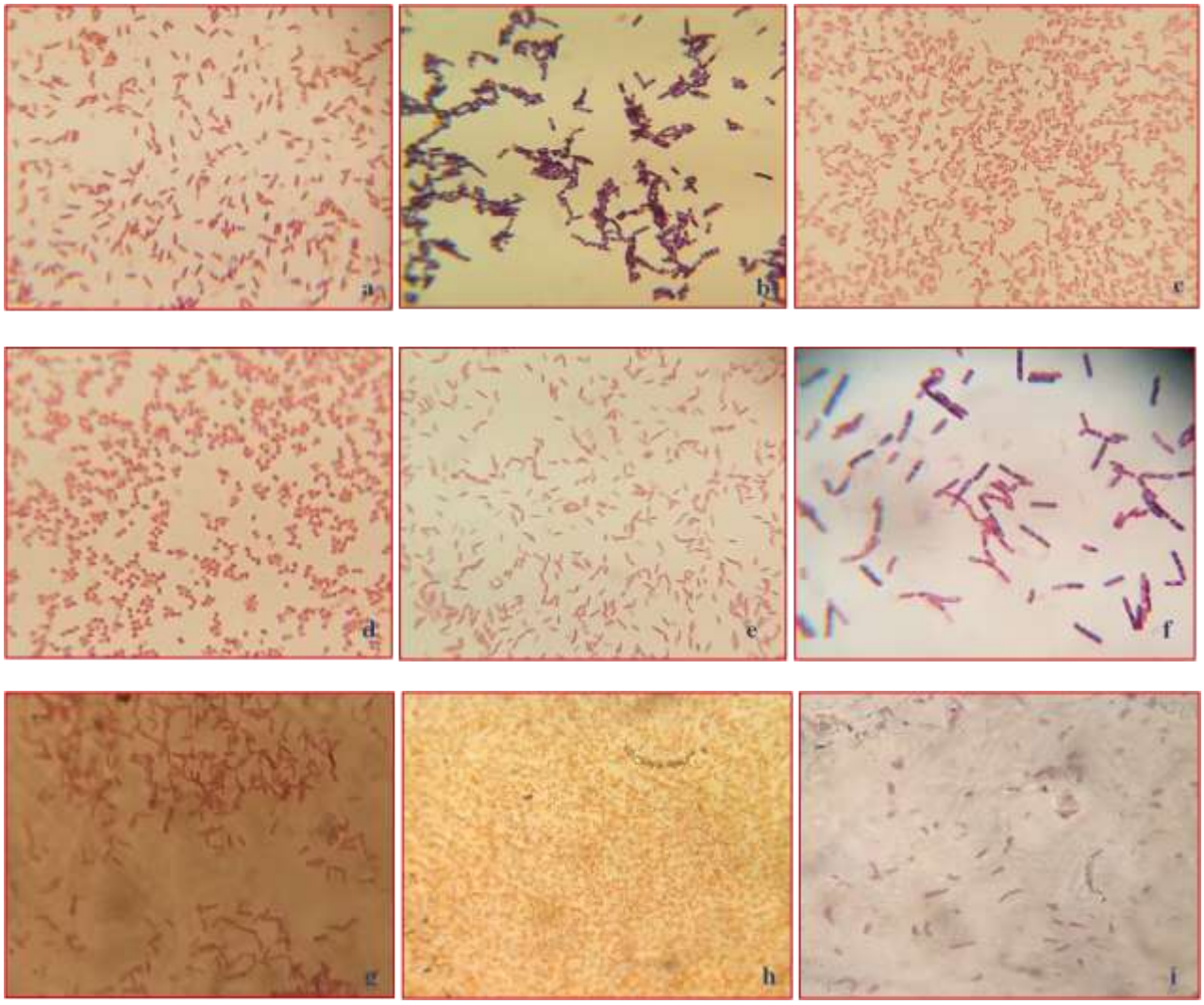


Plate: 4.3 Different morphological appearance of bacterial endophytes isolated from different tissues of Pigeon pea.

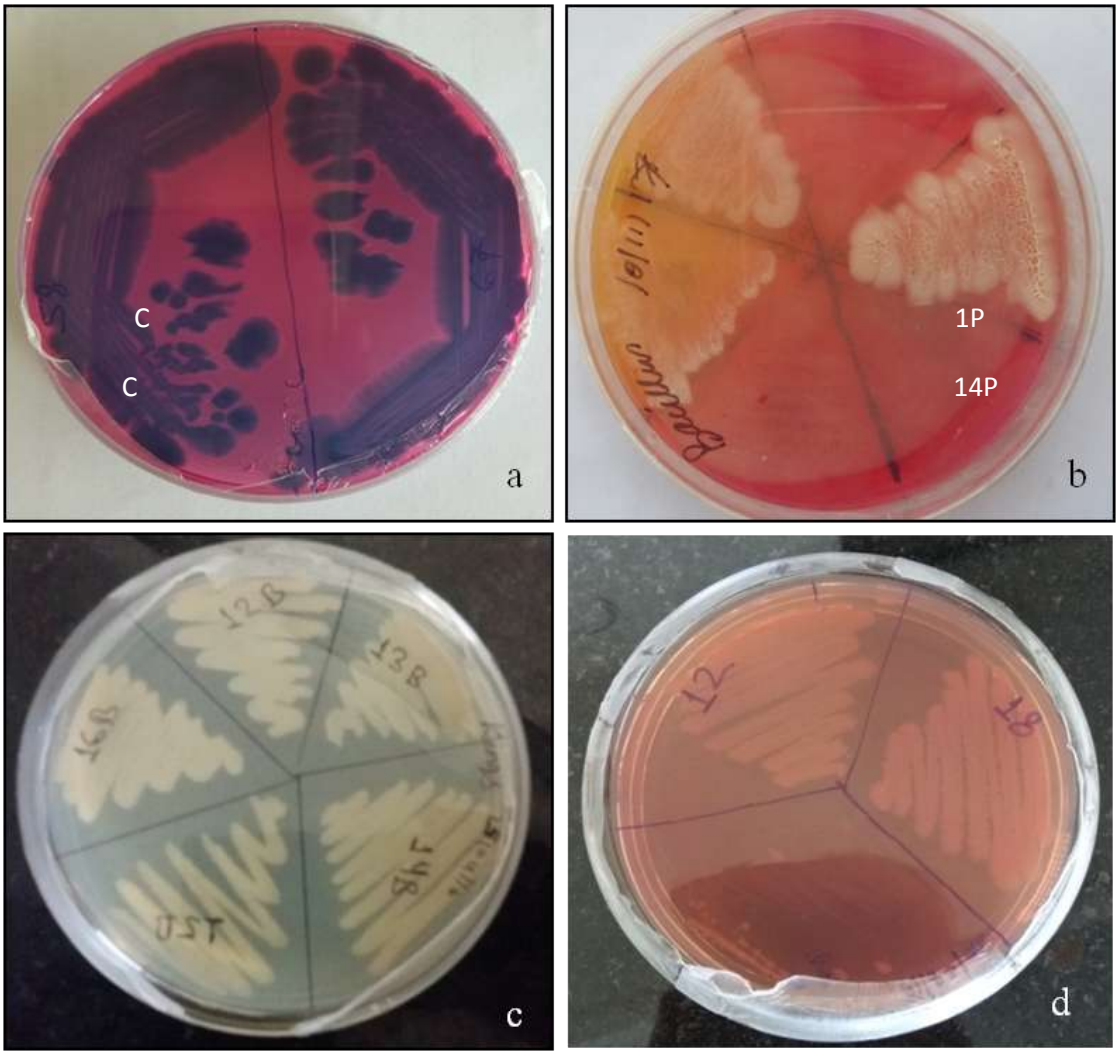


Plate: 4.4 Screening of bacterial endophytes on selective culture medias (Only positive shown) a) Hichrome Bacillus agar plates; b) Bacillus screening plates; c) *Pseudomonas* fleuroscence screening test; and d)Maconkey agar for screening of gram negative bacteria

Table 4.3: Biochemical characterization of bacterial endophytes isolated from Pigeon pea

S. No.	Name of the bacterial endophytic isolates	Gram's reaction	KOH test	Oxidase test	Catalase test	Citrate test	Urease test	Nitrate test	Methyl Red (MR)	Vogus Proskauer (VP)	Motility test	Triple Sugar Iron (TSI) Assay
1.	NIBSM_PR1	+	-	-	+	-	-	+	-	-	Non-motile	OB/OS/NG Blackening
2.	NIBSM_PR2	+	-	-	+	-	-	-	-	-	Non-motile	OB/OS/NG Blackening
3.	NIBSM_PR3	-	+	-	+	+	-	-	-	-	Non-motile	OB/RS/NG
4.	NIBSM_PR4	+	-	-	+	-	-	+	-	-	Motile	YB/YS/NG
5.	NIBSM_PR5	-	+	+	+	+	+	+	-	-	Non-motile	YB/YS/NG B
6.	NIBSM_PR6	+	-	-	-	-	-	-	-	-	Non-motile	OB/OS/NG
7.	NIBSM_PR7	+	-	+	+	+	-	-	-	-	Motile	YB/YS/NG
8.	NIBSM_PR8	-	+	+	-	-	-	-	-	-	Non-motile	YB/YSNG
9.	NIBSM_PR9	+	-	+	+	-	-	-	-	-	Motile	YB/YS/NG
10.	NIBSM_PR10	+	-	-	+	-	-	+	-	-	Non-motile	YB/RS/NG/B
11.	NIBSM_PR11	+	-	+	-	+	+	+	-	-	Non-motile	OB/YS/NG
12.	NIBSM_PR12	+	-	+	-	-	+	+	+	-	Non-motile	RB/YS/NG
13.	NIBSM_PR13	+	-	-	+	+	-	+	-	-	Non-motile	OB/RS/B
14.	NIBSM_PR14	-	+	-	+	+	-	-	-	-	Motile	YB/YS/GP
15.	NIBSM_PR15	+	-	+	+	-	-	-	-	-	Motile	YB/RS/B.

16.	NIBSM_PR16	-	+	-	-	-	-	+	-	-	Non-motile	OB/RS/NG
17.	NIBSM_PR17	-	+	-	+	-	-	+	-	-	Motile	YB/YS\NG
18.	NIBSM_PR18	-	+	-	-	-	+	-	-	+	Motile	YB/RS/NG/B
19.	NIBSM_PR19	-	+	-	+	+	-	-	-	+	Motile	YB/RS/NG/B
20.	NIBSM_PS1	+	-	+	+	-	-	+	+	-	Non-motile	YB/RS/NG/B
21.	NIBSM_PS2	+	-	+	+	+	-	-	+	-	Non-motile	OB/RS/NG
22.	NIBSM_PS3	+	-	+	+	-	-	-	+	-	Motile	OB/RS/NG
23.	NIBSM_PS4	-	+	-	-	+	-	-	-	-	Motile	YB/RS/B
24.	NIBSM_PS5	-	+	-	-	+	+	+	-	-	Motile	YB/RS/GP
25.	NIBSM_PS6	-	+	-	+	+	+	+	-	-	Motile	YB/RS/GP
26.	NIBSM_PS7	+	-	+	+	+	-	+	-	-	Motile	RS/YB/NG
27.	NIBSM_PS8	-	+	-	+	+	-	+	+	-	Motile	YB/YS/NG
28.	NIBSM_PS9	-	+	-	-	-	-	-	-	-	Motile	YB/YS/NG
29.	NIBSM_PS10	+	-	+	+	-	-	-	-	-	Non-motile	YB/RS/NG
30.	NIBSM_PS11	+	-	+	-	+	+	-	+	-	Motile	OB/OS/NG
31.	NIBSM_PL1	+	-	+	+	+	-	-	-	-	Non-motile	OB/YS/NG
32.	NIBSM_PL2	+	-	+	+	-	-	-	-	-	Non-motile	YB/YS/NG
33.	NIBSM_PL3	+	-	+	+	+	-	-	-	-	Non-motile	OB/RS/NG

34.	NIBSM_PL4	+	-	+	+	-	-	-	-	-	Motile	OB/YS/NG
35.	NIBSM_PL5	+	-	+	+	-	-	+	-	-	Motile	YB/YS/NG
36.	NIBSM_PL6	+	-	+	+	+	-	-	-	-	Non-motile	OB/OSR/NG
37.	NIBSM_PL7	+	-	+	-	+	+	-	-	-	Non-motile	YB/OS/NG
38.	NIBSM_PL8	+	-	+	-	-	-	-	-	-	Non-motile	OB/YS/NG
39.	NIBSM_PL9	-	+	-	+	+	-	+	-	-	Non-motile	OB/RS/NG
40.	NIBSM_PL10	-	+	-	+	-	-	+	-	-	Motile	OB/OS/NG
41.	NIBSM_PL11	-	+	-	-	+	+	-	-	+	Motile	YB/RS/GP
42.	NIBSM_PF1	+	-	+	-	-	+	-	+	-	Motile	OB/OSR/NG
43.	NIBSM_PF2	+	-	+	+	-	-	-	+	-	Non-motile	OB/OS/NG
44.	NIBSM_PP1	+	-	+	-	-	-	-	-	-	Non-motile	OB/YS/NG
45.	NIBSM_PP2	-	+	-	+	+	-	+	+	-	Motile	YB/YS/NG
Total Positive		**28**	**17**	**24**	**30**	**21**	**10**	**18**	**9**	**3**	**22**	

Abbreviations: OB- Orange bud; RS- Red Slant; G-No gas

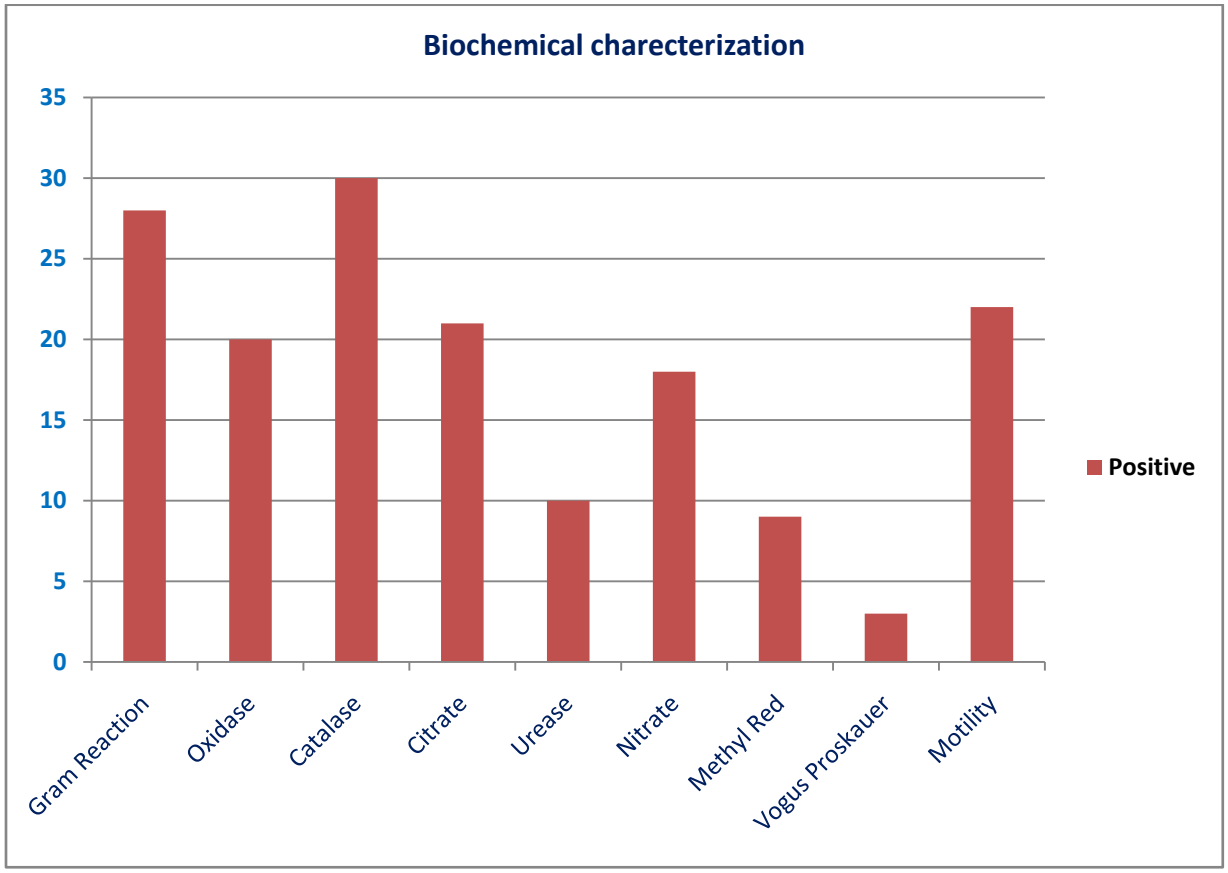


Figure: 4.1 Graphical representation of biochemical activities exhibited bythe bacterial endophytes

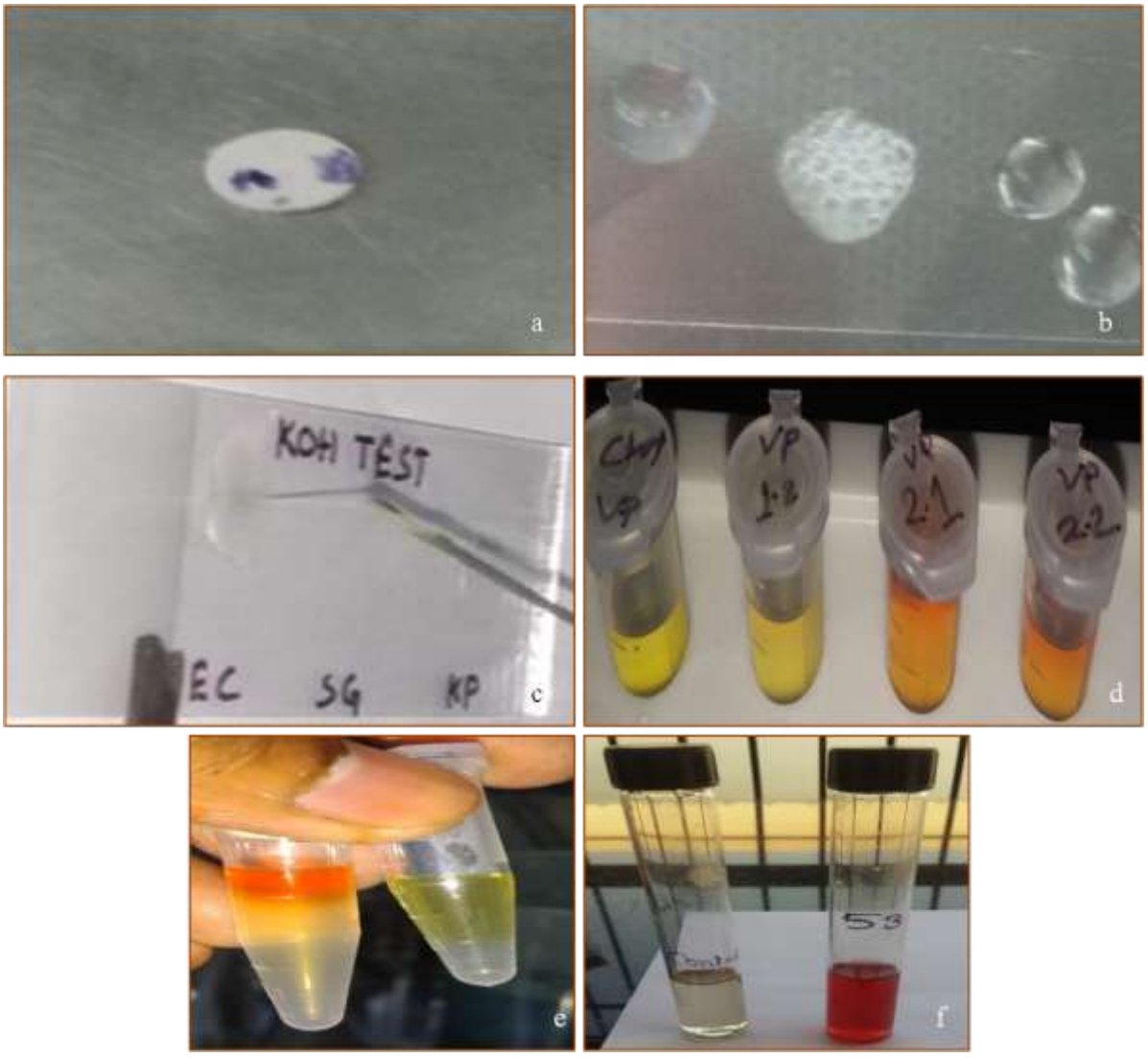


Plate: 4.5 Biochemical characterization of bacterial endophytes (Only positive shown)
a) Oxidase test; b) Catalase test; c) KOH test; d)Voges–Proskauer testa; e) Methyl Red test;and f) Nitrate reduction test

Plate: 4.6 Triple sugar iron (TSI) test forbacterial endophytes (Only culture showing change in colour and gases produce are shown)

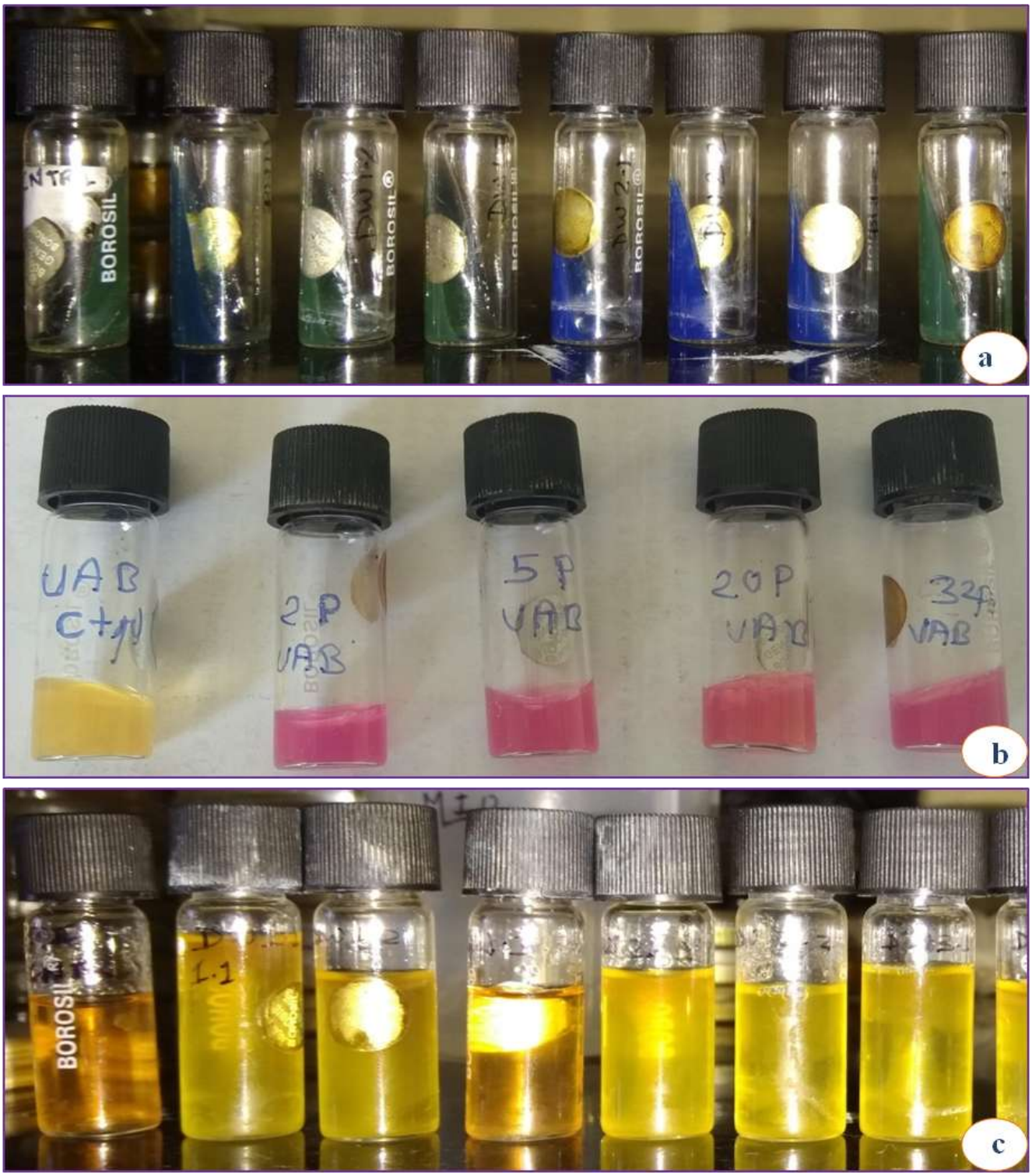


Plate: 4.7 a) Simmon citrate; b) UAB test; and c) Motility test on MIU media (Only shown-positive culture)

Bacteria endophytic from plant roots and nodules of fieldpea and pigeonpea grown in Northern India were isolated. A total of seventy five (75) endophytic bacteria roots and nodules of field pea (Narula *et al.,* 2013) and eighty eight (88) from roots and nodules of pigeonpea showed that fifty (50%) in roots and 93.4% in nodules were gram positive and most of the isolates were spore formers (Saini *et al.,* 2013).

Furthermore, Kumar *et al.,* (2013) isolated a total of 136 nodule and 90 root endophytic bacterial isolates from roots and nodules of legumes and roots of non-legumes crops namely, chickpea (*Cicer arietinum*), field pea (*Pisum sativum*), and lucerne (*Medicago sativa*) and non-legumes wheat (*Triticum aestivum*) and oat (*Avena sativa*). The bacterial diversity was estimated and found that the higher number of Gram positive bacteria were present in legume nodules than in its roots and reported the presence of 7 bacterial genotypes in legume roots and 6 bacterial genotypes in non-legume roots. About 47.8% bacterial endophytes from legume roots and 56 % from roots samples were solubilizing phosphate (P). Based on sequencing of 16S rRNA gene sequencing *Bacillus subtilis* strain CNE 215, *Bacillus licheniformis* strain CRE 1 and *Bacillus flexus* were identified from chickpea root nodule, root and wheat root, respectively.

4.4 Molecular characterization of endophytic bacterial isolates of pigeon pea.

4.4. Isolation of genomic DNA and qualitative and quantitative analysis

The genomic DNA from (45) bacterial endophytes was isolated and it was used to analyzed for quality and quantity using spectrophotometer (Table 4.4)showed the purity and concentration of DNA ranged from 53.64 to 494.75 ng/µl in bacterial isolate NIBSM_PP1 and NIBSM_PR7, respectively. Agarose gel electrophoresis showed the single intact band of genomic DNA in all the isolates (Plate 4.8). The genomic DNA of isolates was further used for PCR amplification of 16S rDNA region.

PCR amplification of 16S rDNA genes its sequencing and NCBI analysis

PCR amplification of 16S rDNA region using forward (27 F) and reverse (1492 R), primers produced ~ 1500 bp amplicon size from all the isolates. The amplified PCR products were sequenced bi-directionally using forward and/ or reverse primers. The nucleotide sequences generated by sequencing were used BLAST analsysis at NCBI database. On the basis of maximum homology, the isolates were identified. The 16S rDNA gene sequences of all the isolates were in the Gene Bank, NCBI database. The detail of molecular characterization and

identification of bacterial endophytes using 16S rDNA along with NCBI accession numbers is given in the Table 4.5**.**

In order to identify the tissue specific bacterial endophytes, a comparison was made to group the endophytes which are commonly present among the tissues and have only their presence in particular tissues of pigeonpea. It was found that the 2 (*Bacillus megaterium, Bacillus aryabhattai)* isolates were common in leaf and stem, while one each isolates were found common among leaf, pod and stem (*Pantoea dispersa*), root and stem (*Beijerinckia fluminensis*) leaf and root (*Bacillus tropicus*), flower and root (*Enterobacter cloacae*). The number of isolates exclusively present in different tissues was 11, 3,4,1,1 in the root, stem, leaf, flower, pod tissues, respectively. The detail of common and exclusive bacterial endophytes found in the different plant tissues depicted in the Table 4.6and Figure 4.2.

Molecular based techniques were applied in various studies involving the characterization of microbes including bacterial endophytes. The results are in accordance with the previous report of Palaniappan *et al.* (2010) obtained thirty nine (39) strains of bacterial endophytic from the nodule of *Lespedeza sp.* grown in two different locations of South Korea and strains were also characterized by using small 16S rRNA as *Arthrobacter, Bacillus, Bradyrhizobium, Burkholderia, Dyella, Methylobacterium, Microbacterium, Rhizobium and Staphylococcus*.

4.5 Screening of endophytic bacterial isolates of pigeon pea for multifarious PGP traits

4.5.1 Indole acetate acid (IAA) production

IAA is one of the most important and physiological active auxin and it increases root surface area and length, therefore provides greater access to soil nutrients and water uptake. A total 45 endophytic bacterial isolates were tested for qualitative IAA production of which 4 bacterial endophytes were found IAA positive (Table 4.7and Plate 4.9).

IAA production allows the bacteria with competitive advantages to colonize plant tissuesas bacterial IAA loosens plant cell wall and promotes root exudation providing additional nutrients to support the growth of bacteria. Indole acetic acid may function as a key signal molecule in the regulation of host plant root development and enhancing the plant microbe interaction.

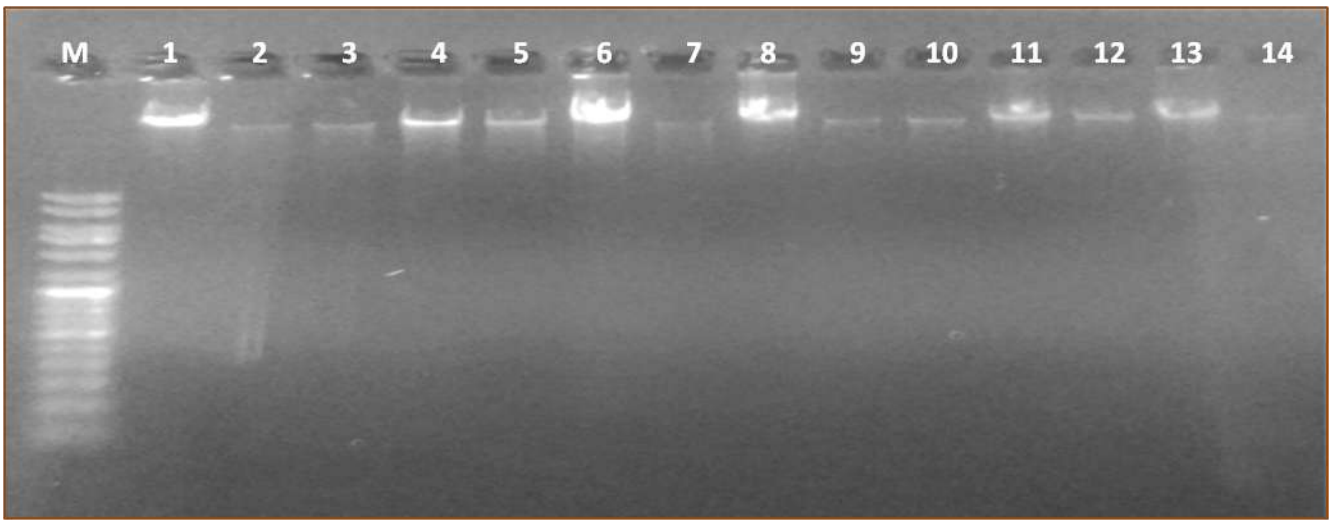
4.5.2 Phosphate solubilization

Among the 45 endophytic bacterial isolates screened for their phosphate solubilizing activity on Pikovosky media of which (11 Nos) 24% were positive and showed a clear yellow

Table 4.4: Qualitative and quantitative analysis bacterial DNA using spectrophotometer

S. No.	Name of the bacterial endophytic isolates	260/ 280 ratio	Concentration (ng/µl)
1.	NIBSM_PR1	1.72	256.72
2.	NIBSM_PR2	1. 65	94.99
3.	NIBSM_PR3	1.68	231.54
4.	NIBSM_PR4	1.62	126.36
5.	NIBSM_PR5	1.78	155.71
6.	NIBSM_PR6	1.58	116.91
7.	NIBSM_PR7	1.72	494.75
8.	NIBSM_PR8	1.78	113.24
9.	NIBSM_PR9	1.74	177.06
10.	NIBSM_PR10	1.59	138.90
11.	NIBSM_PR11	1.71	216.39
12.	NIBSM_PR12	1.79	92.89
13.	NIBSM_PR13	1.69	73.48
14.	NIBSM_PR14	1.71	143.49
15.	NIBSM_PR15	1.79	108.53
16.	NIBSM_PR16	1.71	67.41
17.	NIBSM_PR17	1.74	63.36
18.	NIBSM_PR18	1.66	65.48
19.	NIBSM_PR19	1.71	74.11
20.	NIBSM_PS1	1.65	68.51
21.	NIBSM_PS2	1.72	55.96
22.	NIBSM_PS3	1.67	104.12
23.	NIBSM_PS4	1.62	126.94
24.	NIBSM_PS5	1.72	138.95
25.	NIBSM_PS6	1.64	205.65

26.	NIBSM_PS7	1.72	136.65
27.	NIBSM_PS8	1.57	100.51
28.	NIBSM_PS9	1.62	61.23
29.	NIBSM_PS10	1.27	72.01
30.	NIBSM_PS11	1.34	148.01
31.	NIBSM_PL1	1.39	354.61
32.	NIBSM_PL2	1.57	108.13
33.	NIBSM_PL3	1.32	221.22
34.	NIBSM_PL4	1.50	88.00
35.	NIBSM_PL5	1.65	421.75
36.	NIBSM_PL6	1.30	118.4
37.	NIBSM_PL7	1.61	190.0
38.	NIBSM_PL8	1.69	55.12
39.	NIBSM_PL9	1.47	65.78
40.	NIBSM_PL10	1.21	449.63
41.	NIBSM_PL11	1.71	89.17
42.	NIBSM_PF1	1.77	110.97
43.	NIBSM_PF2	1.06	219.77
44.	NIBSM_PP1	1.82	53.64
45.	NIBSM_PP2	1.64	93.25



Gel image showing genomic DNA isolated from bacterial endophytes

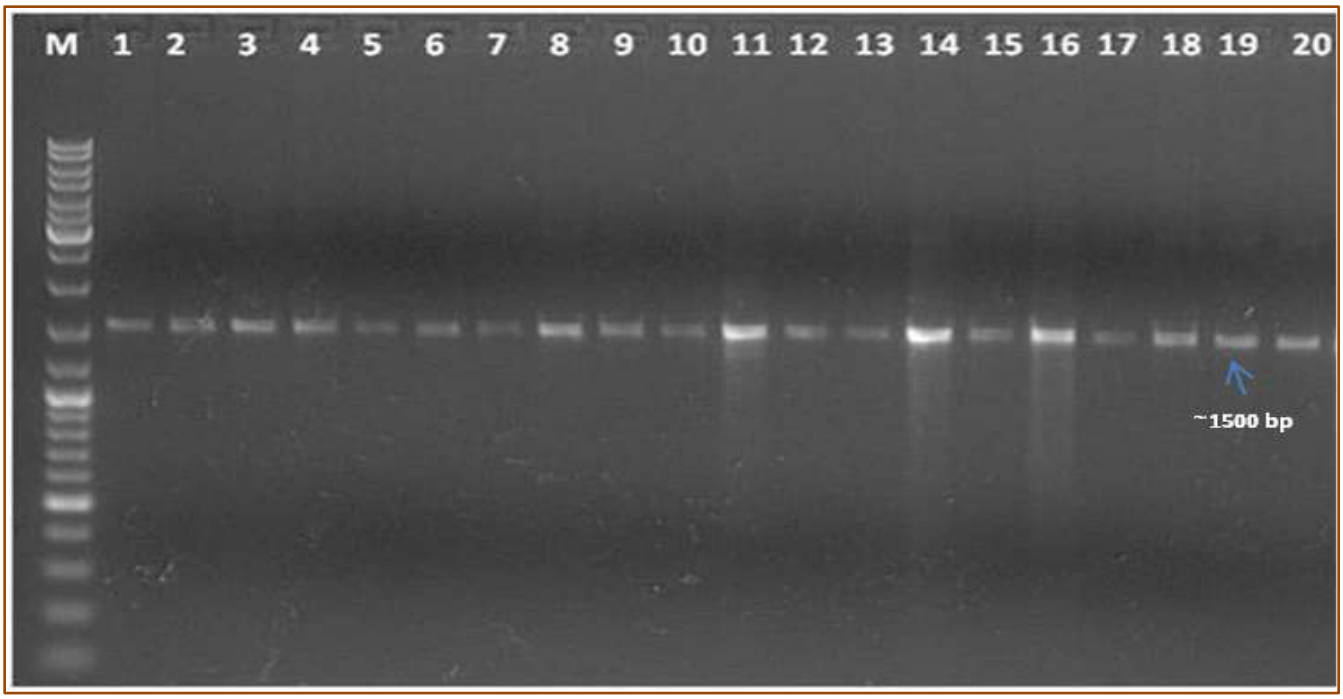


Plate: 4.8 Gel image showing PCR amplification of 16S rDNA fragment from bacterial endophytes isolated from Pigeon pea

Table 4.5: Molecular characterization and identification of bacterial endophytes isolated from Pigeonpea

S.No	Bacterial Isolate/ Strain	Name of the Bacterial Endophytes	Isolation source	Length (bp)	Matching with NCBI database including accession number	% Similarity	Accession Number
1.	NIBSM_PR1	*Bacillus velezensis*	Root	810	*Bacillus velezensis* strain FZB42 16S ribosomal RNA, complete sequence [NR_075005.2]	99%	MK342206
2.	NIBSM_PR2	*Bacillus amyloliquefaciens*	Root	821	*Bacillus amyloliquefaciens* strain MPA 1034 16S ribosomal RNA gene, partial sequence [NR_075005.2]	99%	MK342207
3.	NIBSM_PR3	*Pseudomonas geniculata*	Root	918	P*seudomonas geniculata* ATCC 19374 = JCM 13324 16S ribosomal RNA, partial sequence [NR_024708.1]	99%	MK342208
4.	NIBSM_PR4	*Bacillus tropicus*	Root	956	*Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence [NR_157736.1]	99%	MK342209
5.	NIBSM_PR5	*Klebsiella oxytoca*	Root	976	*Klebsiella oxytoca* strain ATCC 13182 16S ribosomal RNA gene, partial sequence [NR_041749.1]	99%	MK342210
6.	NIBSM_PR6	*Fictibacillus phosphorivorans*	Root	821	*Fictibacillus phosphorivorans* strain Ca7 16S ribosomal RNA gene, partial sequence [NR_118455.1]	99%	MK342211
7.	NIBSM_PR7	*Bacillus paramycoides*	Root	883	*Bacillus paramycoides* strain MCCC 1A04098 16S ribosomal RNA, partial sequence [NR_157734.1]	99%	MK342212
8.	NIBSM_PR8	*Herbaspirillum aquaticum*	Root	830	*Herbaspirillum aquaticum* strain IEH 4430 16S ribosomal RNA gene, partial sequence [NR_116605.1]	99%	MK342213
9.	NIBSM_PR9	*Bacillus tropicus*	Root	928	*Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence [NR_157736.1]	99%	MK342214
10.	NIBSM_PR10	*Bacillus velezensis*	Root	634	*Bacillus velezensis* strain FZB42 16S ribosomal RNA, complete sequence [NR_075005.2]	99%	MK342215
11.	NIBSM_PR11	*Beijerinckia fluminensis*	Root	928	*Beijerinckia fluminensis* strain UQM 1685 16S ribosomal RNA gene, partial sequence [NR_116306.1]	99%	MK342216
12.	NIBSM_PR12	*Beijerinckia fluminensis*	Root	782	*Beijerinckia fluminensis* strain UQM 1685 16S ribosomal RNA gene, partial sequence [NR_116306.1]	99%	MK342217
13.	NIBSM_PR14	*Enterobacter xiangfangensis*	Root	878	*Enterobacter xiangfangensis* strain 10-17 16S ribosomal RNA gene, partial sequence [NR_126208.1]	99%	MK342219

14.	NIBSM_PR15	*Bacillus tropicus*	Root	792	*Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence [NR_157736.1]	99%	MK342220
15.	NIBSM_PR16	*Pseudomonas entomophila*	Root	400	*Pseudomonas entomophila* strain L48 16S ribosomal RNA, partial sequence [NR_102854.1]	100%	MK342221
16.	NIBSM_PR17	*Enterobacter cloacae*	Root	390	*Enterobacter cloacae* strain ATCC 13047 16S ribosomal RNA, complete sequence [NR_102794.2]	96%	MK342222
17.	NIBSM_PR18	*Enterobacter cloacae subsp. Dissolvens*	Root	400	*Enterobacter cloacae subsp. dissolvens* strain ATCC 23373 16S ribosomal RNA gene, partial sequence [NR_118011.1]	99%	MK342223
18.	NIBSM_PR19	*Fictibacillus phosphorivorans*	Root	528	*Fictibacillus phosphorivorans* strain Ca7 16S ribosomal RNA gene, partial sequence [NR_118455.1]	100%	MK342224
19.	NIBSM_PS1	*Bacillus aryabhattai*	Stem	468	*Bacillus aryabhattai* strain B8W22 16S ribosomal RNA gene, partial sequence [NR_115953.1]	99%	MK327147
20.	NIBSM_PS2	*Serratia marcescens*	Stem	538	*Serratia marcescens* strain NBRC 102204 16S ribosomal RNA gene, partial sequence [NR_114043.1]	99%	MK327148
21.	NIBSM_PS3	*Bacillus megaterium*	Stem	546	*Bacillus megaterium* strain ATCC 14581 16S ribosomal RNA gene, partial sequence [NR_116873.1]	99%	MK327149
22.	NIBSM_PS4	*Serratia marcescens*	Stem	830	*Serratia marcescens* strain NBRC 102204 16S ribosomal RNA gene, partial sequence [NR_114043.1]	99%	MK327150
23.	NIBSM_PS5	*Serratia marcescens*	Stem	772	*Serratia marcescens* strain NBRC 102204 16S ribosomal RNA gene, partial sequence [NR_114043.1]	99%	MK327151
24.	NIBSM_PS6	*Bacillus aerius*	Stem	840	*Bacillus aerius* strain 24K 16S ribosomal RNA gene, partial sequence [NR_118439.1]	99%	MK327152
25.	NIBSM_PS7	*Pantoea dispersa*	Stem	790	*Pantoea dispersa* strain DSM 30073 16S ribosomal RNA gene, partial sequence [NR_116797.1]	99%	MK327153
26.	NIBSM_PS8	*Herbaspirillum frisingense*	Stem	587	*Herbaspirillum frisingense* strain NBRC 102522 16S ribosomal RNA gene, partial sequence [NR_114140.1]	87%	MK327154
27.	NIBSM_PS9	*Serratia marcescens*	Ste	830	*Serratia marcescens* strain NBRC 102204 16S ribosomal RNA gene, partial sequence [NR_114043.1]	99%	MK327155

28.	NIBSM_PS10	*Serratia marcescens*	Stem	773	*Serratia marcescens* strain JCM 1239 16S ribosomal RNA gene, partial sequence [NR_113236.1]	99%	MK327156
29.	NIBSM_PS11	*Beijerinckia fluminensis*	Stem	382	*Beijerinckia fluminensis* strain UQM 1685 16S ribosomal RNA gene, partial sequence [NR_116306.1]	99%	MK327157
30.	NIBSM_PL1	*Bacillus aryabhattai*	Leaf	596	*Bacillus stratosphericus* strain 41KF2a 16S ribosomal RNA gene, partial sequence [NR_042336.1]	100%	MK332317
31.	NIBSM_PL2	*Bacillus stratosphericus*	Leaf	840	*Bacillus aerius* strain 24K 16S ribosomal RNA gene, partial sequence [NR_118439.1]	99%	MK332318
32.	NIBSM_PL3	*Bacillus aryabhattai*	Leaf	702	*Bacillus aryabhattai* strain B8W22 16S ribosomal RNA gene, partial sequence [NR_115953.1]	99%	MK332319
33.	NIBSM_PL4	*Bacillus zhangzhouensis*	Leaf	596	*Bacillus zhangzhouensis* strain MCCC 1A08372 16S ribosomal RNA, partial sequence [NR_148786.1]	95%	MK332320
34.	NIBSM_PL5	*Bacillus megaterium*	Leaf	772	*Bacillus megaterium* strain ATCC 14581 16S ribosomal RNA gene, partial sequence [NR_116873.1]	99%	MK332321
35.	NIBSM_PL6	*Bacillus megaterium*	Leaf	445	*Bacillus megaterium* strain ATCC 14581 16S ribosomal RNA gene, partial sequence [NR_116873.1]	98%	MK332322
36.	NIBSM_PL7	*Bacillus aryabhattai*	Leaf	576	*Bacillus aryabhattai* strain B8W22 16S ribosomal RNA gene, partial sequence [NR_115953.1]	99%	MK332323
37.	NIBSM_PL8	*Bacillus tropicus*	Leaf	762	*Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence [NR_157736.1]	99%	MK332324
38.	NIBSM_PL9	*Pseudomonas stutzeri*	Leaf	420	*Pseudomonas stutzeri* strain ATCC 17588 16S ribosomal RNA, complete sequence [NR_103934.2]	99%	MK332325
39.	NIBSM_PL10	*Pantoea dispersa*	Leaf	460	*Pantoea dispersa* strain DSM 30073 16S ribosomal RNA gene, partial sequence [NR_116797.1]	99%	MK332326
40.	NIBSM_PL11	*Pseudomonas plecoglossicida*	Leaf	586	*Pseudomonas plecoglossicida* strain NBRC 103162 16S ribosomal RNA gene, partial sequence [NR_114226.1]	98%	MK332327
41.	NIBSM_PF1	*Enterobacter cloacae subsp. Dissolvens*	Flower	538	*Enterobacter cloacae subsp. dissolvens* strain ATCC 23373 16S ribosomal RNA gene, [NR_118011.1]	99%	MK327244
42.	NIBSM_PF2	*Bacillus malikii*	Flower	586	*Bacillus malikii* strain NCCP-662 16S ribosomal RNA,	97%	MK327245

					partial sequence [NR_146005.1]		
43.	NIBSM_PP1	*Paenibacillus polysaccharolyticus*	Pod	567	*Paenibacillus polysaccharolyticus* strain BL9 16S ribosomal RNA, partial sequence [NR_108250.1]	97%	MK327269
44.	NIBSM_PP2	*Pantoea dispersa*	Pod	644	*Pantoea dispersa* strain DSM 30073 16S ribosomal RNA gene, partial sequence [NR_116797.1]	99%	MK327270
45.	NIBSM_PP2	*Pantoea dispersa*	Pod	644	*Pantoea dispersa* strain DSM 30073 16S ribosomal RNA gene, partial sequence [NR_116797.1]	99%	MK327270

4.6: List of bacterial endophytes found common and exclusive among the tissues of Pigeon pea (On the basis of 16 S rRNA gene sequencing)

Names of the plant tissues	Total number of bacterial endophytes shared/ unique	Name of the bacterial endophytes
Leaf, Pod, Stem	1	*Pantoea dispersa*
Root, Stem	1	*Beijerinckia fluminensis*
Leaf, Root	1	*Bacillus tropicus*
Flower, Root	1	*Enterobacter cloacae subsp. Dissolvens*
Leaf, Stem	2	*Bacillus megaterium, Bacillus aryabhattai*
Root	11	*Pseudomonas geniculata, Bacillus paramycoides Herbaspirillum aquaticum, Bacillus amyloliquefaciens, Bacillus velezensis, Enterobacter cloacae, Klebsiella oxytoca, Enterobacter xiangfangensis, Bacillus subtilis Pseudomonas entomophila, Fictibacillus phosphorivorans*
Stem	3	*Serratia marcescens, Bacillus aerius, Herbaspirillum frisingense*
Leaf	4	*Pseudomonas plecoglossicida, Bacillus zhangzhouensis, Pseudomonas stutzeri, Bacillus stratosphericus*
Flower	1	*Bacillus malikii*
Pod	1	*Paenibacillus polysaccharolyticus*

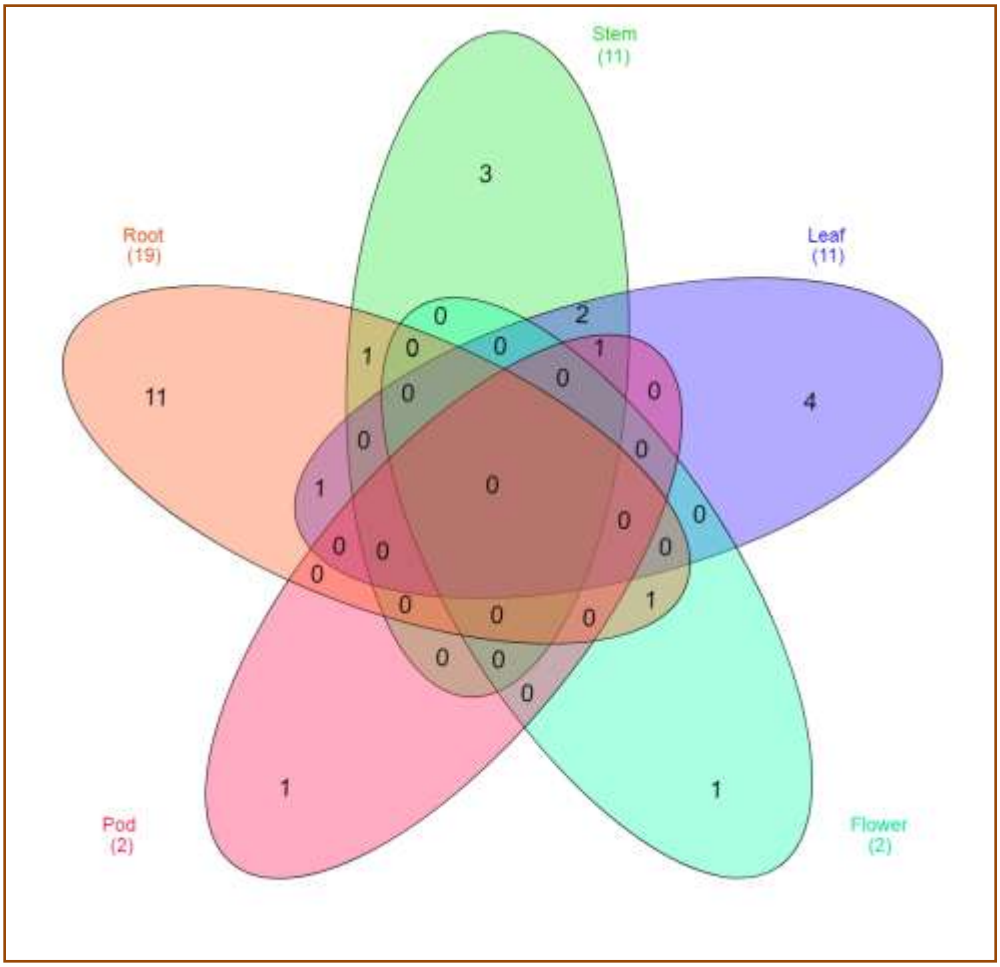
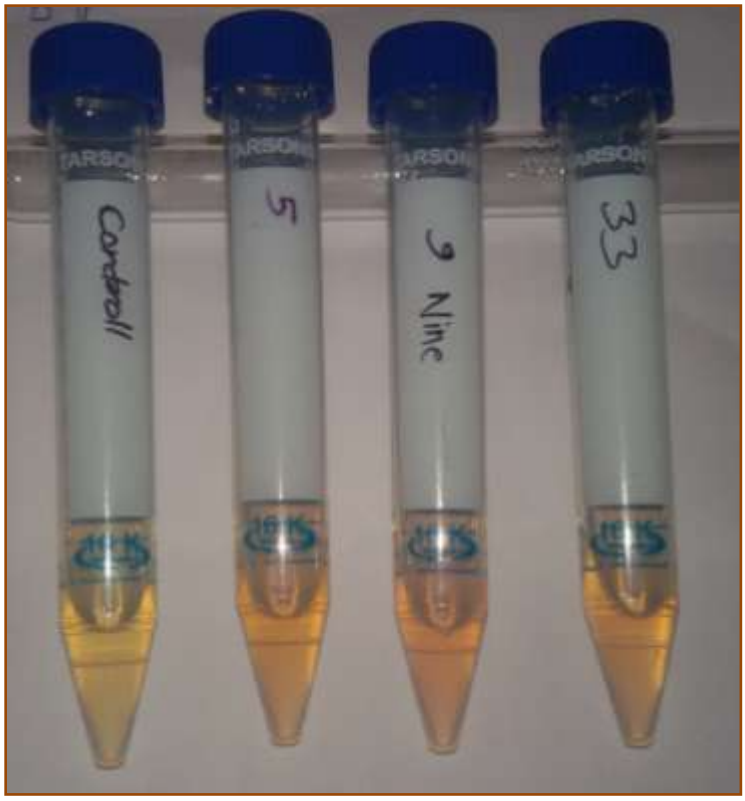


Figure: 4.2 Venn diagram showing common and exclusive bacterial endophytes among the tissues of Pigeon pea

Table 4.7: Plant growth promoting activities (PGP) exhibited by bacterial endophytes

S. No	Bacterial isolates	Phosphate solubilization assay	DNase Assay	IAA Production	Siderophore formation	HCN production
1.	NIBSM_PR1	-	+	-	+	-
2.	NIBSM_PR2	-	+	-	+	-
3.	NIBSM_PR3	-	+	-	+	-
4.	NIBSM_PR4	-	+	-	-	-
5.	NIBSM_PR5	-	-	-	-	-
6.	NIBSM_PR6	-	-	-	+	-
7.	NIBSM_PR7	-	+	-	+	-
8.	NIBSM_PR8	-	-	-	+	-
9.	NIBSM_PR9	-	-	+	-	-
10.	NIBSM_PR10	-	-	-	-	-
11.	NIBSM_PR11	-	-	-	+	-
12.	NIBSM_PR12	-	-	-	+	-
13.	NIBSM_PR13	-	+	-	+	-
14.	NIBSM_PR14	+	-	+	-	-
15.	NIBSM_PR15	-	+	-	+	-
16.	NIBSM_PR16	-	-	-	-	-
17.	NIBSM_PR17	-	-	-	-	-
18.	NIBSM_PR18	+	-	-	-	-
19.	NIBSM_PR19	-	+	-	+	-
20.	NIBSM_PS1	-	-	-	-	-
21.	NIBSM_PS2	-	-	-	-	-
22.	NIBSM_PS3	-	-	-	-	-
23.	NIBSM_PS4	+	+	-	+	-
24.	NIBSM_PS5	+	-	-	-	-

25.	NIBSM_PS6	-	+	-	+	-
26.	NIBSM_PS7	+	-	-	+	-
27.	NIBSM_PS8	+	-	-	-	-
28.	NIBSM_PS9	-	-	-	+	-
29.	NIBSM_PS10	-	+	-	-	-
30.	NIBSM_PS11	-	-	-	+	-
31.	NIBSM_PL1	-	-	-		-
32.	NIBSM_PL2	-	-	-	-	-
33.	NIBSM_PL3	-	+	+	-	-
34.	NIBSM_PL4	-	-	-	-	-
35.	NIBSM_PL5	-	-	-		-
36.	NIBSM_PL6	-	-	-		-
37.	NIBSM_PL7	+	-	-	-	-
38.	NIBSM_PL8	-	-	-	-	-
39.	NIBSM_PL9	+	+	+	+	-
40.	NIBSM_PL10	+	-	-	-	-
41.	NIBSM_PL11	-	-	-	+	-
42.	NIBSM_PF1	-	-	-	-	+
43.	NIBSM_PF2	+	-	-	+	-
44.	NIBSM_PP1	-	-	-	-	-
45.	NIBSM_PP2	+	-	-		-
Total Positive		**11**	**13**	**4**	**19**	**1**



IAA production assay

Plate: 4.9 Plant growth promoting activities IAA showed by bacterial endophytes

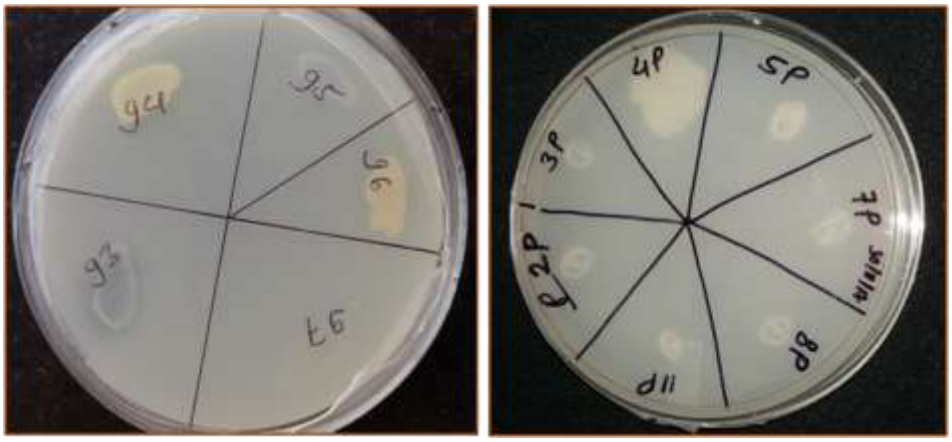


Plate: 10 Phosphate solublization assay

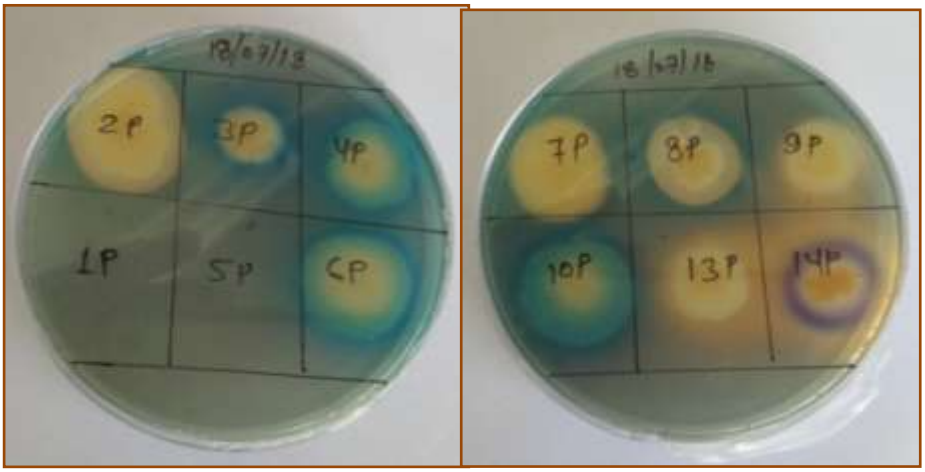


Plate: 11 Siderophore production

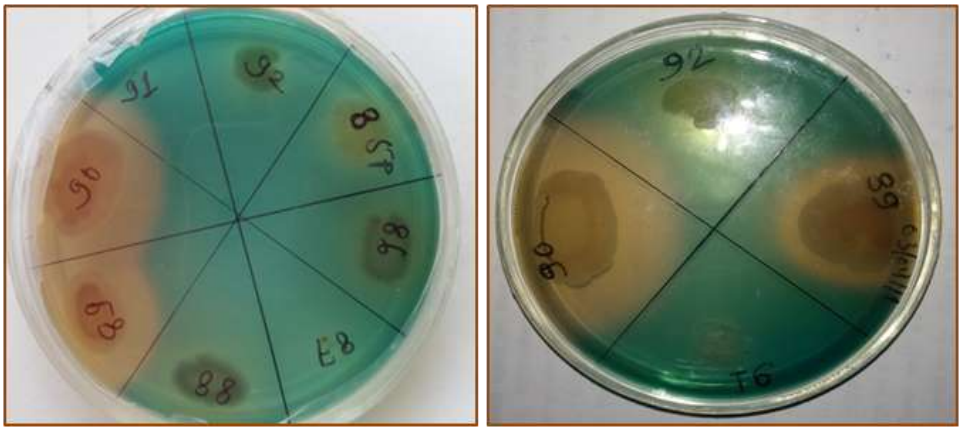


Plate: 12 DNase activities assay

Plant growth promoting positive activities phosphate solubilisation, siderosphore and DNase activities showed by bacterial endophytes.

halo zone around colony on medium ((Table 4.7 and Plate 4.10). The phosphorus solubilizing bacteria were belonging to the genera *Bacillus, Enterobacter, Pantoea* and *Serretia.*

Phosphorus is one of the essential macronutrient and plays an important role in plant growth and in many physiological activities such as cell division, photosynthesis and development of root system. These results are in harmony with Aarab *et al.* (2015) reported the mineral phosphate solubilization activity of four *Pseudomonas* isolates from rice rhizosphere. Plants normally take P from soil as phosphate anions (H_2PO_4 and HPO_4-) and the ability of bacterial isolates to solubilize insoluble phosphate thereby, enhancing the P availability for plant growth.

4.5.3 Siderophore production

Out of 45 endophytic bacterial isolates, (19 nos) 42.2% showed development of orange halo zone on Chrome-azurol S (CAS) medium indicated siderophore production (Plate 4.11) Siderophore is one of the traits that make microorganism successful competitors in various environments and facilitate plant bacterial association as well as colonization of roots, stem and leaf thus, make iron unavailable to pathogenic microorganism. Iron is required in large abundance, as it is involved in various important biological processes, such as photosynthesis, respiration, chlorophyll biosynthesis and biological nitrogen fixation.

Sansanwal *et al.,* (2018) isolated total 41 endophytes from Mungbean only twelve cultures were found positive for siderophore production. These results are in accordance with Kumar *et al*., (2013) who reported out of 150 bacterial isolates 16 were found positive for siderophore production on CAS medium. Similarly, Catherine *et al*., (2012) showed that out of 43 endophytic bacteria, 12 were able to produce orange halo zone on CAS medium and belongs to genera *Pseudomonas sp* and *Flavimonas oryzihabitans.* Our results are in line with finding of Zhao *et al.,* (2011) who demonstrated two endophytes *viz.Bacillus cereus* from *Sophora alopecuroides* root nodules were able to produce siderophores on CAS medium. Similarly, Naureen *et al.,* (2015) reported that out of 63 bacterial isolates from the rhizosphere and rhizoplane of rice and maize plants, 20 were found positive for siderophore production on CAS medium

Production of siderophore by the bacterial strain is one of the biocontrol mechanisms. The Fe chelation by bacteria strain makes them better opponents for the available iron and hence, prevents growth of the phytopathogenic micro-organisms. (Jasim *et al*. 2013). Plant growth

promoting endophytes bacteria also solubilize insoluble soil P to make them available to enhance crop productivity. (Pandey *et al.*, 2006) also reported four endophytic strains namely B*acillus sp, B. cereus, .B. pumilus* and *Pseudomonas putida* which solubilize phosphate.

4.5.4 DNase test

DNase test was conducted for detecting deoxyribonuclease activity in the isolated bacterial endophytes. Out of 45 endophytic bacterial isolates only (13) 28.8% isolates showed DNase activity on DNase base agar media (Table 4.7 and Plate 4.12). DNase assay is also useful for identification of pathogenic Staphylococci, differentiation and identification of non-pigmented *Serretia* species isolated from clinical/environmental sources that might be improperly identified as *Enterobacter* and *Klebsiella* species.

4.5.5 HCN production

Endophytic bacterial isolate separately streaked on glycerin agar medium showed change in color of filter paper from yellow to light brown for moderate (brown) or strong (reddish-brown) indicated HCN production(Table 4.7). Out of 45 only one bacterial isolates was found to have HCN production ability. The results are in agreement with Sansanwal *et al*.(2018) isolated about 41 endophytes from Mungbean and 12 bacterial isolates and identified HCN producing isolates.

4.6 Antagonistic activity

4.6.1 Pigeon pea endophytes bacteria against *Sclerotium rolfsii*

A total of 45 isolated bacterial endophytes were tested for antagonistic activities under *invitro* using dual culture assay. Of which (19) 42.7% of endophytic bacterial isolates showed antagonistic activity against soil borne fungus *Sclerotium rolfsii* and made clear zone of inhibition and inhibited the growth of fungus**.** Bacterial endophytic isolates NIBSM-PR1, NIBSM-PR2, NIBSM-PR11 and NIBSM-PR13 showed better inhibition of fungal growth. The details of isolate wise antagonistic activities are presented in Table 4.8 and Plate 4.13.

4.6.2 Pigeon pea endophytes bacteria against *Fusarium spp*

Antagonistic effect of 45 bacterial endophytic isolates was screened for their antagonism against *Fusarium spp*, of which (14) 31.1% of endophytic bacteria showed antagonistic activity against *Fusarium spp* Table 4.8 and Plate 4.14. The bacterial endophytes showing NIBSM_PR1, NIBSM_PR12, NIBSM_PR19 isolated from root and NIBSM_PL8 from leaf while none of the isolates from stem, flower and pod tissues showed antagonistic activity against *Fusarium.*

Table 4.8: Antagonistic potential of bacterial endophytes against soil borne fungal pathogens

S. No.	Name of the Bacterial isolates	*Sclerotium rolfsii*	*Fusarium sps*	*Rhizoctonia solani*
1.	NIBSM_PR1	+++	+++	+++
2.	NIBSM_PR2	+++	++	+++
3.	NIBSM_PR3	+	+	-
4.	NIBSM_PR4	-	-	++
5.	NIBSM_PR6	+	++	+
6.	NIBSM_PR7	-	-	++
7.	NIBSM_PR8	-	-	+++
8.	NIBSM_PR10	++	-	+++
9.	NIBSM_PR11	+++	+++	+++
10.	NIBSM_PR12	+++	+++	+++
11.	NIBSM_PR13	+++	+	-
12.	NIBSM_PR14	+	++	-
13.	NIBSM_PR15	+	+	-
14.	NIBSM_PR19	-	+++	-
15.	NIBSM_PS7	-	-	++
16.	NIBSM_PS11	+	-	-
17.	NIBSM_PL1	++	++	++
18.	NIBSM_PL3	++	+	+
19.	NIBSM_PL4	++	++	-
20.	NIBSM_PL5	++	-	++
21.	NIBSM_PL6	++	-	+
22.	NIBSM_PL7	+	-	-

23.	NIBSM_PL8	++	+++	-
24.	NIBSM_PL9	++	-	-
25.	NIBSM_PL11	-	-	+
26.	NIBSM_PF1	-	-	+
27.	NIBSM_PP1	-	-	++
Total positive		**19**	**14**	**17**

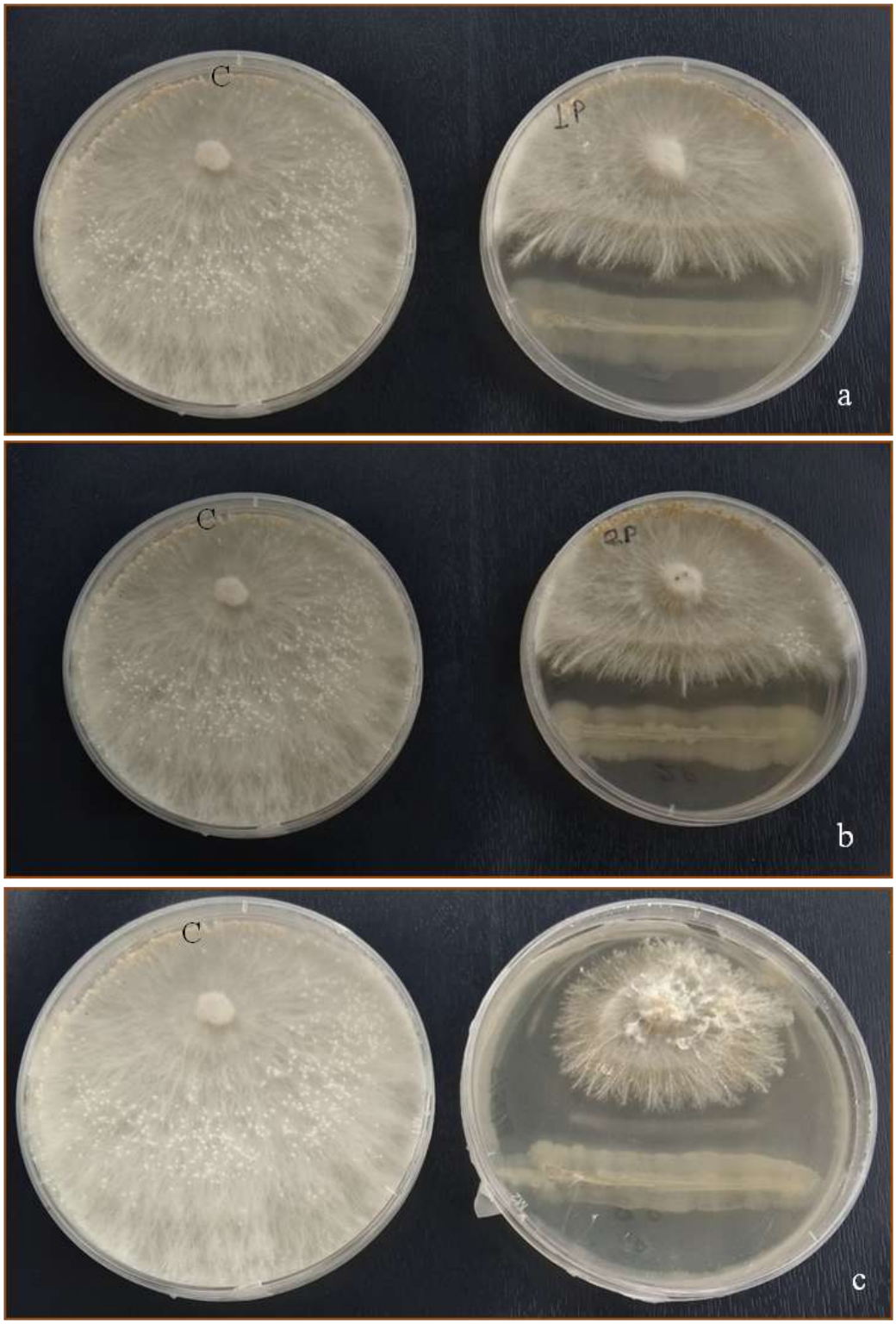


Plate: 4.13 Bacterial endophytes showing antagonistic activities against *Sclerotium rolfsii* Pigeon pea isolate

4.6.3 Pigeon pea endophytes bacteria against *Rhizoctonia solani*

A total of 45 isolated bacterial endophytes were tested for antagonistic potential against *Rhizoctonia solani,* of which (17) 37.7% of endophytic bacterial isolates showed antagonistic activity against *R. solani* Table 4.7.The numbers of isolates from different plant tissues namely root, stem, leaf, flower and pod tissue were 9, 5, 1, 1 and 1, respectively. The bacterial endophytes NIBSM_PR1, NIBSM_PR2, NIBSM_PR8, NIBSM_PR10, isolated from root and NIBSM_PL7 and none of the isolates from stem NIBSM_PS7 and flower NIBSM_PF1 and pod NIBSM_PP1showed antagonistic activity against *Rhizoctonia solani.*

Several bacterial endophytes exhibited antibiotic properties thatprevent the growth of an antagonistic bacterial cell. In this study, bacterial endophytic isolates exhibited strong antagonistic activities against the important fungal pathogens, *Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium spp.* It is assumed that the antifungal activity of the isolates may be due to secretions like production of lytic enzymes, chitinase, lipopeptides and production of certain antibiotics. *Bacillus* spp. has been reported to produce various lytic enzymes including chitinases which degrades chitin, a key component of the fungal cell wall (Kumar *et al.* 2012; Castillo *et al.* 2013). Biotic stress has adverse impacts on plants, including co-evolution, population dynamics, ecosystem nutrient cycling, natural habitat ecology, and horticultural plant health (Gusain *et al.*, 2015). Plants inoculated by soaking their roots or seeds overnight in cultures of PGPR exhibit enormous resistance to different forms of biotic stress (Ngumbi and Kloepper; 2016).

4.7Antibiotic sensitivity pattern of the bacterial endophytes

A total of 45 endophytic bacterial isolates were screened for antibiotics sensitivity test using 14 numbers of multispectral antibiotic discs namely Ampicilin (AMP), Streptomycin (S), Polymyxin (PB), Cifixime (CFM), Vancomycin (VA), Gatifloxacin (GAT), Methicillin (MET), Ciprofloxacin (CIP) , Ceftriaxone (CTR), Tetracycline (TE), Nalidixic acid (NA), Gentamicin (GEN), Trimethoprim (TR), Azithromycin (AZM). Antibiotic sensitivity pattern of the endophytic isolate was determined by disc diffusion method. The completion zone was observed around the discs and measured. On the basis of standard chart sensitive (S) intermediate (I), and resistant (R) to particular antibiotics were recorded. The detail of antibiotic sensitivity is mentioned and Figure 4.3 and Plate 4.15.

Among the bacterial endophytes isolated from pigeonpea showed highest resistance to Ceftriaxone followed by Cifixime, Methicillin, Polymyxin and Ampicilin. Whereas the bacterial strains found highest sensitivity to antibiotics Gatifloxacin followed by Gentamicin. The strains were sensitive to antibiotic Gatifloxacin followed by Gentamicin whereas most of them were resistant to Ceftriaxone followed by Cifixime, Methicillin, Polymyxin and Ampicilin. Amongst all bacterial strains were sensitive to Gatifloxacin but the strain *Beijerinckia fluminensis* (NIBSM_PR11), *Bacillus tropicus* (NIBSM_PR15) isolated from root and *Pseudomonas plecoglossicida* (NIBSM_PL11) isolated from leaf tissues showed resistance to Gatifloxacin.

The results are in agreement with the previous study in turmeric Christina *et al.* (2013) showed that the bacterial endophytes were resistant to rifampicin and polymixin-B and mostly sensitive to chloramphenicol followed by erythromycin.

Many natural products obtained from microbial endophytes have proven to be antibacterial, antifungal, antidiabetic, antioxidants and immunosuppressive and great source of bioactive natural products. The majority of endophytic bacterial isolates are known to produce different kinds of novel antibiotics like Ecomycins, Pseudomycins, Munumbicins, Kakadumycins. These compounds inhibit the growth of pathogenic bacteria and fungi

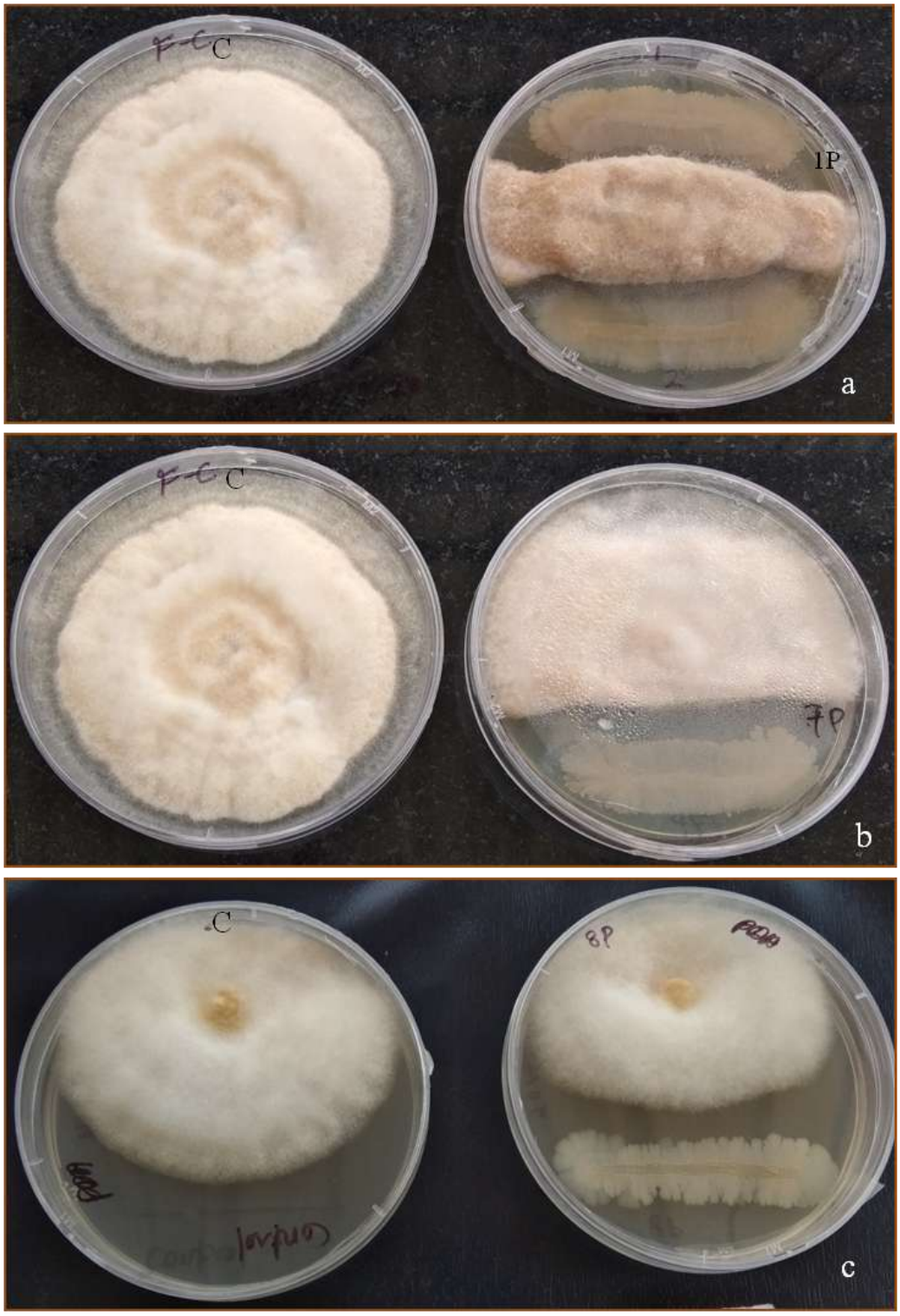


Plate: 4.14 Bacterial endophytes showing antagonistic activities against *Fusarium sps* Pigeon pea isolate

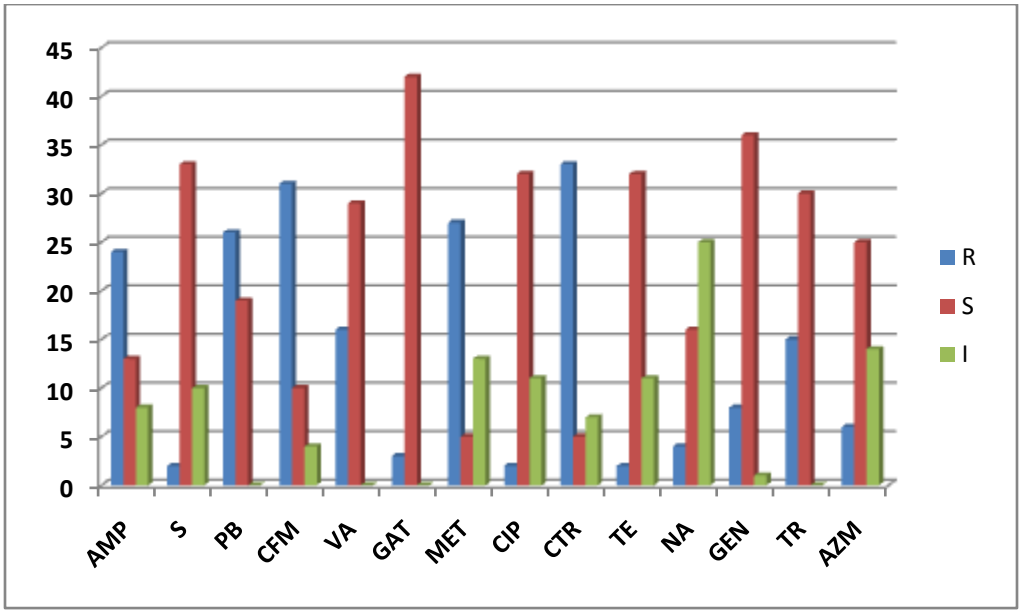


Figure: 4.3 Graphical representative antibiogram of bacterial endophytes isolated from different tissues of Pigeon pea

Abbreviations: AMP-Ampicilin; S-Streptomycin; PB – Polymyxin; CFM - Cefixime; VA-Vancomycin GAT-Gatifloxacin; MET- Methicilin; CIP-Ciprofloxacin CTR- Ceftiaxone; TE-Tetracycline NA-Nalidixic acid GEN-Gentamicin; TR-Trimethoprim AZM-Azithromycin.

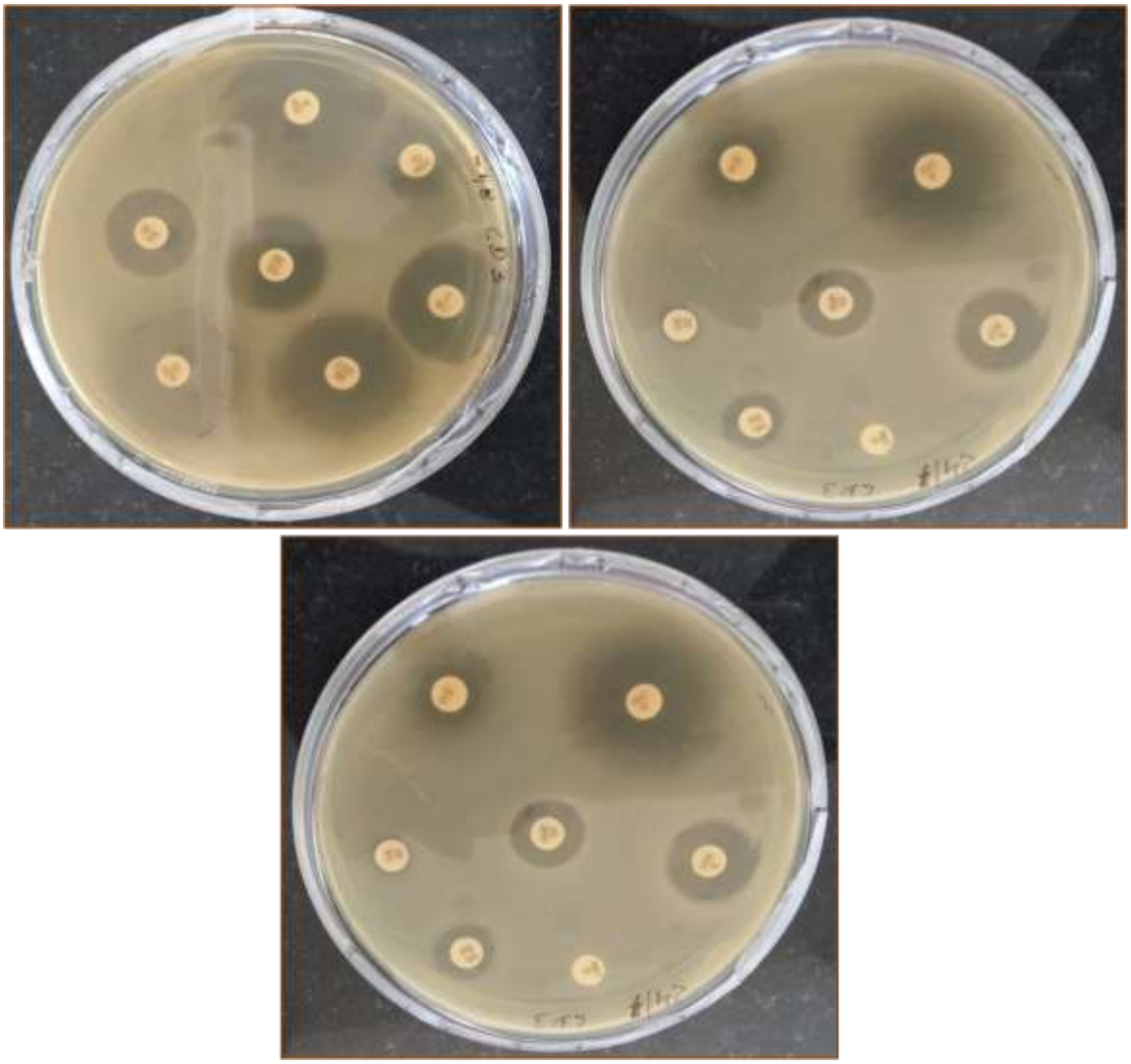


Plate: 4.15. Antibiotics sensitivity test of bacterial endophytes isolated from Pigeon pea

CHAPTER-V
SUMMARY AND CONCLUSION

The present investigation entitled "Isolation and characterization of endophytic bacteria from pigeon pea (*Cajanus cajan* L.)" was carried out at ICAR- National Institute of Biotic Stress Management, Baronda, Raipur (Chhattisgarh)

Endophytes are the microbe which colonize and live inside the plant tissues and organs have positive impact on plant growth and health. Bacterial endophytes have been reported to have larger influence on the performance, growth and stress tolerance of host plants. Endophytes initially enter the plant endosphere, adapt new environment and overcome plant defense response and their distribution within plant depends on their ability to colonize and the allocation of plant resources. Keeping in keep the importance of bacterial endophytes, the present study was conduct to isolate and identify potential bacterial endophytes from pigeonpea and their screening for beneficial traits such as plant growth promoting (PGP) and antagonistic activities against fungal pathogens.

A total of 45 bacterial endophytes were isolated from seven pigeonpea lines and five plant tissues namely root, stem, leaf, flower and pods. The number of isolates isolated tissue wise (19) 42.2%, (11) 24.4%, (11) 24.4%, (2) 4.4% and (2) 4.4% from root, stem, leaf, flower and pod/seed tissues, respectively. Out of 45 isolates, 38 bacterial isolates, representing from root (15 nos), stem (11) and leaf (8), flower (2) and pod (2) were isolated from six recommended varieties (PL-1 to PL-6) of pigeon pea, while 7 bacterial isolates comprising root (4) and leaf (3) from pigeonpea line Raipur local (PL-7 were isolated.

Morphological and colony characteristics of bacterial endophytes showed that the small to medium sized irregular shape and the size of most of the isolated varied from pin to medium. Most of the bacterial endophytes (36) have individual rod shape and (8) cocco-bacilli and coccii, while (1) bacteria had capsulated form. Gram's reaction of bacterial isolates showed that the (28) of bacterial isolates were Gram positive and (17) were gram negative.

Biochemical characterization showed that the number of isolates was positive for Oxidase (24), Catalase (30), Citrate utilization (21), Methyl Red (9) and Vogus Proskauer (3), Urease (10), Nitrate reduction (18). The motility and gases producing ability of isolates showed that 22 were motile and (6) isolates produced gas.

Molecular characterization of bacterial endophytes was performed by amplification and sequencing of 16S rDNA gene. On the basis of nucleotide sequences generated by sequencing of 16S rDNA amplicon bacterial isolates were identified and grouped in different genera's namely *Bacillus, Fictibacillus, Pseudomonas, Enterobactor, Klebsiella*, *Beijerinckia*, *Pantoea,* and *Serretia.* The gene sequences of all the 45 bacterial endophytes have been deposited to Genbank, NCBI and their accession numbers were obtained.

Endophytes having plant growth promoting (PGP) activities are needed not only for the agronomic improvement of agricultural crops but also for their subsequent impact in enhancing tolerance to diseases *via* growth enhancement. A favorable endophyte-host association in crop plants would minimize the usage of agricultural inputs, such as fertilizer and pesticides, thus saving costs and reducing pollutants to the environment.

The bacterial isolates were screened for their plant growth-promoting (PGP) activities viz., production of indole acetic acid (4), siderophore (19), phosphate solubilization (11), HCN production (1) were found positive. Thus the endophytes identified with potential plant growth promoting activities can be used as bio fertilizer and for further molecular studies.

DNase test was conducted for detecting deoxyribonuclease activity in the isolated bacterial endophytes. Out of 45 endophytic bacterial isolates only (13) 28.8% isolates showed DNase activity on DNase base agar media. DNase assay is also useful for identification of pathogenic Staphylococci, differentiation and identification of non- pigmented *Serretia* species isolated from clinical/environmental sources that might be improperly identified as *Enterobacter* and *Klebsiella* species.

Endophytes are well known to produce antimicrobial substances, secondary metabolites and other small peptides. Bacterial isolates were screened for antibiotics sensitivity pattern using 14 numbers of multispectral antibiotics showed that highest resistance to Ceftriaxone followed by Cifixime, Methicillin, Polymyxin and Ampicilin, while highest sensitivity to Gatifloxacin followed by Gentamicin.

Antagonistic activity test identified bacterial isolates having antagonism against soil borne fungal pathogens *viz, Sclerotium rolfsii, Fusarium sps,* and *Rhizoctonia solani*.

A total of 45 isolated bacterial endophytes were tested for antagonistic activities under *invitro* using dual culture assay. Of which (19) 42.7% of endophytic bacterial isolates showed antagonistic activity against soil borne fungus *Sclerotium rolfsii* and made clear zone of

inhibition and inhibited the growth of fungus**.** Bacterial endophytic isolates NIBSM-PR1, NIBSM-PR2, NIBSM-PR11 and NIBSM-PR13 showed better inhibition of fungal growth.

Fourteen (31.1%) bacterial endophytes isolates showed antagonistic activities against *Fusarium spp,* the endophytes showing antagonism namely NIBSM_PR1, NIBSM_PR12, NIBSM_PR19 were isolated from root and NIBSM_PL8 from leaf. However, none of the isolates from stem, flower and pod tissues showed antagonistic activity against *Fusarium.*

A total of 45 isolated bacterial endophytes were tested for antagonistic potential against *Rhizoctonia solani,* of which (17) 37.7% of endophytic bacterial isolates showed antagonistic activity against *R. solani*. The numbers of isolates from different plant tissues namely root, stem, leaf, flower and pod tissue were 9, 5, 1, 1 and 1, respectively. The bacterial endophytes NIBSM_PR1, NIBSM_PR2, NIBSM_PR8, NIBSM_PR10, isolated from root and NIBSM_PL7 and none of the isolates from stem NIBSM_PS7 and flower NIBSM_PF1 and pod NIBSM_PP1showed antagonistic activity against *Rhizoctonia solani*

Highest antagonistic activities against soil borne fungal pathogens were exhibited by bacterial endophytes isolated from root tissues. The potential isolates exhibiting antifungal activities may be used for the development of biocontrol formulations for controlling multiple biotic stresses.

Suggestions for future work

On the basis of microbial resources and information generated from this study, the future research work may be planned as follows:

- Bacterial endophytes isolated during the study could be used for *in planta* validation of plant growth promoting activities and their probable role in improving plant nutrition and plant growth enhancement.
- Bacterial endophytes having antagonism against soil borne fungal pathogen need to be further validated on pot experiment.
- Identification of molecular mechanism and factors (metabolites, enzyme etc.) providing defense against soil borne fungal pathogens.

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APPENDIX- I

1. Chemicals and glassware's

1. Chemical and glassware used were procured from the following sources:
2. Himedia Biosciences, India.
3. Eppendorf India Ltd., India.
4. Thermo fisher Ltd, India.
5. Tarsons Products Pvt. Ltd., India.

2. Composition of Media and supplements

I. Potato dextrose agar

Ingredients	g / liter
Potato dextrose agar	200
Dextrose	20.0
Distilled water	1000 ml
pH	6.8

II. Composition of Nutrient Agar

Ingredients	g / l
Peptone	5.0
NaCl	5.0
K2HPO4	1.5
MgSO4.7H2O	1.5
Agar	15.0
Distilled water	1000 ml
Final pH	7.0±0.2

III. Nutrient Broth (NB)

Ingredients	g / l
Peptone	5.0
Yeast extract	1.5
Beef extract	1.5
NaCl	5.0
Distilled water	1000 ml
pH	7.0

IV. **Composition of Pikovaskaya‟s medium (Pikovaskaya 1948)**

Ingredients	g / l
Glucose	10.0
Ca3 (PO4)2 –	5.0
(NH4)2SO4	0.5
NaCl	0.2
MgSO4.7H20	0.1
KCl	0.2
Yeast extract	0.5
MnSO4.7H20	0.002
FeSO4.7H20	0.002
Agar	20 .0
pH	7.0
Distilled Water	1000 ml

V. Nitrate reduction test reagent:

Ingredients	g / l
Solution A: Sulphanilic acid	8.0
Acetic acid (5N)	1000 ml
Solution B :	
α Naphthylamine	5.0
Acetic acid	10–00 ml

VI. Methyl Red (MR) reagent:

Ingredients	g / l
Methyl Red	0.02g
Ethanol (95%)	60 ml
Distilled water	40 ml

VII. VP broth (gl^{-1})

Ingredients	g / l
Peptone	7.0
Glucose	5.0
Potassium phosphate	5.0

pH	6.9

VIII. **Voges-Proskauer (VP) tests reagents:**

Reagent A:

Ingredients	g / l
Alpha- naphthol	5.0
Ethanol (95%)	100 ml

Reagent B:

Ingredients	g / l
KOH	40.0
Creatine	0.3

IX. King's B medium

Ingredients	g / l
Proteose peptone	20.0
Glycerol	10 ml
K2HPO4	1.5
MgSO4	1.5
Agar	16.0
H2O	1000 ml
pH	7.0

X. Simmon's citrate agar

Ingredients	g / l
Sodium citrate	0.2
Magnesium sulphate	0.02
Ammonium dihydrogen Phosphate	0.1
Agar	15
pH	6.8.7.2

XI. Triple sugar agar

Ingredients	g / l
Beef extract	3.000
Peptone	20.000
Yeast extract	3.000

Lactose	10.000
Sucrose	10.000
Dextrose monohydrate	1.000
Ferrous sulphate	0.200
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.00

XII. Bacillus Medium

Ingredients	g / l
L-Glutamic acid	4.0
Glycerol	15.0 ml
K2HPO4	2.5
MgSO4.7H2O	6.0
Agar	20.0
Distilled water	1000 ml
pH	7.2

XIII. Urease agar base

Ingredients	g / l
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000
Monopotassium phosphate	2.000
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.8±0.2

XIV. Mullar hinton agar

Ingredients	g / l
Beef extract	2.0
Acid hydrolase of casein	17.05
Starch	1.50

Agar	17.00
Distilled water	1000 ml

XV. Hichrome Bacillus media

Ingredients	g / l
Peptic digest of animal tissue	15.00
Chromogenic mixture	2.500
Agar	13.50
Casein enzymic hydrolysate	4.000
Final pH (at 25°C)	7.2±0.2

XVI. Composition of stains

Gram Stain Solutions

Crystal violet solution

Crystal violet	10 g
Ammonium oxalate	4 g
Ethanol	100 ml
Distilled water	400 ml

Iodine solution

Iodine	1 g
Potassium iodide	2 g
Ethanol	25 ml
Distilled water	100 ml

Alcohol

Distilled water	5 ml
Ethanol	95 ml

Counter stain

2.5% Safranin	10 ml
Distilled water	100 ml

Salkowaski reagent

12 g $FeCl_3$ in 1 liter H_2SO_4 (7.9M)

Saline Water

8.5 g NaCl in 1000 ml water

APPENDIX II

1. Solutions and reagents used for molecular biology work

Reagent/Stock solutions, their composition and method of preparation

S. No.	Reagent/Stock solution	Method of preparation
1.	1 M Tris HCl (pH 8.0)	Tris base 121.1 g Distilled water 800 ml HCl 42 ml Final volume 1000 ml
2.	0.5 M EDTA (pH 8.0)	Disodium EDTA. $2H_20$ 186.1g Distilled water 800 ml NaOH 20 g Final volume 1000 ml
3.	5 M NaCl	NaCl 292 g water 800 ml Distilled water 800 ml
4.	10 N NaOH	NaOH 400 g water 800 ml Final volume 1000 ml
5.	Potassium acetate buffer	5 M Potassium acetate 60 ml Glacial acetic acid 11.5 ml Final volume 100 ml
6.	SDS Stock (10%)	SDS 100 g Distilled water 900 ml
7.	Phenol: Chloroform	Equal quantity of Tris saturated phenol with chloroform
8.	Ethidium bromide	Ethidium bromide 1g Distilled water 100 ml
10.	Gel Loading dye	Bromophenol blue 0.25% Xylene carol 0.25% Glycerol 30% in water
11.	50 X TAE buffer	Tris base 242 g

		Glacial Acetic acid 57.1 ml 0.5 M EDTA 100 ml Final volume 1000 ml
12.	Ethidium Bromide : Stock 10mg/ml	10 mg Ethidium Bromide 1 ml distilled water

RESUME

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