FIELD EVALUATION OF POTENTIAL FLUORESCENT *PSEUDOMONAS* ISOLATES ON GROWTH AND YIELD ATTRIBUTING CHARACTERS AND INDUCTION OF ANTIOXIDANT ENZYMES IN SOYBEAN, CHICKPEA AND WHEAT

Ph.D. Thesis

by

Rathod Parashuram

DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF AGRICULTURE
FACULTY OF AGRICULTURE
INDIRA GANDHI KRISHI VISHWAVIDYALAYA
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Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

by

Rathod Parashuram

IN PARTIAL FULFILMENT OF THE REQUIRMENTS
FOR THE DEGREE OF

Doctor of Philosophy

in

Plant Pathology

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CERTIFICATE - I

This is to certify that the thesis entitled **"Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters and induction of antioxidant enzymes in soybean, chickpea and wheat"** submitted in partial fulfilment of the requirement for the degree of **"Doctor of Philosophy in Agriculture"** of the Indira Gandhi krishi vishwavidyalaya, Raipur, is a record of the bonafied research work carried out by **Rathod Parashuram** under my guidance and supervision. The subject of thesis has been approved by student's Advisory committee in the Director of Instruction.

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

Date: 29|10|2020

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THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

Chairman	(Dr. A.S. Kotasthane)
Member	(Dr. N. Khare)
Member	(Dr. Satish B. Verulkar)
Member	(Dr. R. R. Saxena)
Head of Department	

CERTIFICATE - II

This is to certify that the thesis entitled **"Field evaluation of potential fluorescent *Pseudomonas* isolates,on growth and yield attributing characters and induction of antioxidant enzymes in soybean, chickpea and wheat"** submitted By **Rathod Parashuram** to the Indira Gandhi krishi vishwavidyalaya, Raipur in partial fulfillment for **Doctor of Philosophy** in the Department of **Plant Pathology** has been approved by external examiner and student's advisory committee after oral examination.

Date: 23/02/2021

Signature External Examiner
(Name:)

Major Advisory ________________________

Head of Department ________________________

Faculty Dean ________________________

Approved/Not approved

Director of Instruction ________________________

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Department of Plant Pathology
College of Agriculture, IGKV
Raipur (CG).

Rathod Parashuram

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LIST OF NOTATIONS AND ABBREVIATIONS

%	Per cent
@	At the rate of
/	Per
^{o}C	Degree Celsius
µg	Microgram
±	Plus minus
Ml	Micro litre
ACCD	1-amino cyclo propane-1 carboxylic acid deaminase
ADW	Autoclaved distilled water
BCP	Bromo cresol purple
CAS	Chrome azurol sulphonate assay
CD	Critical difference
cm	Centimetre
CMA	Chloromolybdic acid
CSA	Chlorostannous acid
CV	Coefficient of variance
DAS	Day after sowing
et al.	And Co-Worker/And Other
Fig.	Figures
IAA	Indole-3 acetic acid
i.e.	That is
M	Molar
mM	Millimolar
ml	Millilitre
mg	Milligram
OD	Optical density
PDA	Potato Dextrose Agar
Rpm	Revolution per minute
PGPR	Plant growth promoting rhizobacteria
PGPA	Plant growth promoting activity
KMB	King’s medium B base

THESIS ABSTRACT

a) Title of the Thesis : **“Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters and induction of antioxidant enzymes in soybean, chickpea, and wheat”**

b) Full Name of the Student : Rathod Parshuram

c) Major Subject : Plant Pathology

d) Name and Address of the Major Advisor : Dr. A. S. Kotasthane, Professor & Head, Department of Plant Pathology

e) Degree to be Awarded : Ph.D. (Ag.) Plant Pathology

Signature of the Student

Signature of Major Advisor

Date: 29-10-20

Signature of Head of the Department

ABSTRACT

The present investigation entitled **Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters and induction of antioxidant enzymes in soybean, chickpea, and wheat”** was initiated with the isolation of fluorescent *Pseudomonas* from the soil and *Brassica* specific root rhizosphere. Eighty-eight rhizosphere soil samples were derived from different locations of Bastar and Bilaspur districts of Chhattisgarh. Four rhizospheric soil samples were derived from *Brassica* spp. from zhahirabad (Telangana). Ten fluorescent *Pseudomonas* isolates (on UV light 360nm) emitting strong fluorescence were selected for the course of the investigation. For fundamental research and practical application of PGPR strains on different crops, we require potential isolates. Our present investigation indicates that beneficial effects of fluorescent *Pseudomonas* can be achieved through seed treatment which is simple and cost-effective. Three fluorescent *Pseudomonas* 9704, BS-4, and 9829 consistently, over different locations, increased seed germination, reduced seedling mortality, improved plant growth and yield attributes in soybean, chickpea, and wheat, and also stimulated enhanced antioxidant activity in chickpea and wheat. Plant growth-promoting effects and an increase in grain yield of fluorescent *Pseudomonas* isolates were crop-specific. Phylogenetic affinities of the 16S rRNA DNA and ITS 27+1492 gene sequences resolved the species identity of our potential isolates. In the present study, we report, a method to raise plantlets of different crops in general and soybean in particular in modified Petri plates and described methodology for *in vitro* interaction (bi and tri-partite interaction) involving root-soil interface in semi-artificial experimental set-ups.

शोध का सारांश

शोध का शीर्षक : “खेत मूल्यांकन प्रक्रिया का उपयोग करके सशक्त फ्लोरोसेंट स्यूडोमोनास आइसोलेट्स का सोयाबीन, चना और गेहूं मे विकास, उपज के कारक और एंटीऑक्सीडेंट एंजाइमो के प्रेरक का अध्ययन किया गया है”

छात्र का नाम : राठौड़ परशुराम

मुख्य विषय : पादप रोग विज्ञान

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(पादप रोग विज्ञान), कृषि महाविद्यालय, इंदिरा गांधी कृषि
विश्वविद्यालय, रायपुर, 492012 (छत्तीसगढ़)

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छात्र का हस्ताक्षर

प्रमुख सलाहकार का हस्ताक्षर

दिनांक 29-10-20

विभागाध्यक्ष का हस्ताक्षर

सारांश

प्रस्तुत अनुसंधान प्रक्रिया जिसका शीर्षक है “खेत मूल्यांकन प्रक्रिया का उपयोग करके सशक्त फ्लोरोसेंट स्यूडोमोनास आइसोलेट्स का सोयाबीन, चना और गेहूं में विकास उपज के कारक और एंटीऑक्सीडेंट एंजाइमो के प्रेरक का अध्ययन किया गया है” का आरंग फ्लोरोसेंट स्यूडोमोनास के आइसोलेशन से किया गया है । फ्लोरोसेंट स्यूडोमोनास को ब्रैसिका विशिष्ट जड़ तथा अट्ठासी विभिन्न मृदाएं जो कि छत्तीसगढ़ राज्य के बस्तर व बिलासपुर संभाग से किया गया है । अथवा चार ब्रैसिका विशिष्ट जड़ मृदा का संलग्न तेलंगाना राज्य के जहीराबाद संभाग

से किया गया है I फ्लोरोसेंट स्यूडोमोनास के 10 आइसोलेट्स को 360 नैनो मीटर के पराबैगनी प्रकाश से उत्सर्जन करके अध्ययन किया गया है। उपरोक्त दस स्यूडोमोनास आइसोलेट्स का उपयोग करके पौधे मे विकास को बढ़ावा देने के नियामक गतिविधि का मौलिक व व्यवहारिक प्रयोग किया गया है| हमने फ्लोरोसेंट स्यूडोमोनास का उपयोग करके बीज उपचार के सरल एवं किफायती तरीके से इस्तेमाल किया है | विभिन्न संभागों में जब 3 किस्म के फ्लोरोसेंट स्यूडोमोनास का परीक्षण किया गया तब यह ज्ञात होता है कि सोयाबीन, चना और गेहूँ की फसलों मे इसकी सहायता से बीज अधिक अंकुरण तथा अंकुरित बीजों के मृत्यु दर में कमी पाई गई, जिसके फलस्वरूप पौधों की वृद्धि तथा उपज के इकाई में बढ़त पाई गई, तथा एंटीऑक्सीडेंट सक्रिय होते हैं उपरोक्त फ्लोरोसेंट स्यूडोमोनास के सशक्त आइसोलेट्स की वंशावली सूची को 16s आरएनए डीएनए तथा आईटी एस 27 + 1492 जीन से अनुक्रम किया गया और सभी आइसोलेट्स को विशिष्ट वर्गों में विभाजित किया गया है | अंततः हमारे अनुसंधान कार्य में अंकुरित बीज के अध्ययन हेतु परिवर्तित पेट्री प्लेट प्रतिमान बनाया गया है जिसकी सहायता से कृतिम स्थिति में (द्वि एवं तीन-पक्षीय पारस्परिक क्रिया) पौधों के जड़ एवं मृदा का इंटरफ़ेस स्थापित किया गया |

CHAPTER-I

INTRODUCTION

The world population is increasing at an alarming rate which 2050 is expected to reach 9.6 billion approaching the estimated planet's maximum capacity (Wilson, 2003). Currently, around 925 million people across the globe are suffering from hunger and malnutrition. It was expected by 2050 almost 2 billion people are going to suffer from the same (Eldakak et al., 2013). A modernized cropping system can help to deal with global food security for the increasing rate of the population that will increase (double) the agricultural production (Bruinsma, 2009). An increase in agricultural production requires additional inputs (i.e. fertilizer pesticides) that will safeguard our crop from infestation by pathogen insects and invading weeds latest technology can help farmers on sustainable agriculture can boost and maintain yield and productivity which can frequently de with changing environment condition (Baulcombe et al., 2009).

Microbial products are now becoming a valuable component in precision agriculture (Nobbe and Hiltner, 1896; Deaker et al., 2004; Copping, 2009) in addition to the new chemistries and trait development which are an important critical component in developing biotic and abiotic stress tolerance/resistance crop (Berg, 2009; Bhattacharyya and Jha, 2012). Reid and Greene, 2012 suggested that microbes can increase agriculture production with the help of sustainable remedies and explained the limitation of microbes to feed the growing population. A wide variety of rhizosphere-inhabiting bacteria can stimulate plant growth by direct or indirect mechanisms. Plant growth-promoting rhizobacteria facilitate plant growth through direct/indirect mechanisms (bio fertilization, stimulation of root growth, rhizoremediation, and plant stress control, reducing the level of disease, antibiosis, induction of systemic resistance, and competition for nutrients and niches) (Lugtenberg and Kamilova 2009; Glick, 2014). Plant roots exudate release a large number of organic compounds (organic acid, phytosiderophore, sugars, vitamins, amino acid, etc) which acts as the signaling compound to the microbial population. This helps to metabolites the root exudates by microbes which increase the microbial population (Bais et al., 2006: Pothier et al., 2007: Badri et al., 2009;

Shukla et al., 2011; Drogue et al., 2013) indicate that the root system and its rhizosphere interact with the biotic environment. The PGPR activity by microbial population imposes an impact on the rhizosphere and the complete plant is still challenging. Bipartite interaction with roots and PGPR in vitro indicates that growth of primary roots may decrease due to PGPR (Dobbelaere et al., 1999), increase in number, and length is due to PGPR (Combes-Meynet et al., 2011; Chamam et al., 2013), also initiates the elongation of root hair *in vitro* (Dobbelaere et al., 1999; Contesto et al., 2008), PGPR inoculated plants shows an increase in root and shoot biomass (El Zemrany et al., 2006; Minorsky, 2008; Veresoglou and Menexes, 2010; Walker et al., 2012). Consequently, the uptake of minerals and water is stimulated by PGPR activity helps in the growth of the complete plant. Beneficial soil microbes showed the capability of inducing antioxidant enzymes such as SOD (superoxide dismutase), POD (peroxidase) which are associated with PGPR mediated induced systemic resistance and protect against several stresses (Younesi and Moradi 2014; Karthikeyan et al. 2006).

Seed bacterization or seed inoculation with PGPR bacteria helps them to establish in soil and rhizosphere (Ma et al., 2016a). Nevertheless, due to the poor survival of bio inoculants, the large-scale commercial production of biofertilizer or biocontrol agents is hindered (Ma et al., 2011). Thus, improving the formulation of microbial inoculum that can survive, establish, and develop in the soil to provide nutrients to plant. Through seed treatment with PGP microbes, complete protection solutions can be provided to seeds against different environmental stresses, which can be cost-effective and nature friendly which leads to precision agriculture (Hazra and Patanjali, 2016). Seed treatment with PGP microbes can be handled more easily and used to protect the plant from biotic stress (like plant pathogens) and abiotic stress (like drought and salinity). Recent developments reveal that coating seed with microbes can efficiently help to inoculate and develop its population in the soil, which significantly produce the microbes coated seed to meet higher safety measure and efficiency standard (Ma et al., 2019; Rocha et al., 2019). Any successful strategy aimed at enhancing crop productivity with microbial products ultimately relies on the ability to scale at regional to global levels. Microorganisms that show promise in the lab may lack key characteristics for widespread adoption in sustainable and productive agriculture systems. To make a significant impact on global sustainable agriculture, the implementation of plant beneficial microorganisms will require a more seamless transition between laboratory and farm applications. The present investigation was therefore undertaken with the following major objectives:-

1. Collection, isolation and biochemical characterization of fluorescent *Pseudomonas* derived from soil and *Brassica* specific root rhizosphere
2. Studies on bipartite and tripartite interaction using potential fluorescent *Pseudomonas*, *Macrophomina phaseolina*, and soybean model system
3. Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters of soybean, chickpea, and wheat following seed treatment.
4. Antioxidant enzyme assays of chickpea and wheat plants derived from a seed treatment with potential fluorescent *Pseudomonas* and molecular characterization of potential isolates.

CHAPTER-II

REVIEW OF LITERATURE

2.1 Fluorescent *Pseudomonas*

Plant root surrounded by a thin layer of soil supports large groups of bacteria known as plant-growth-promoting rhizobacteria (PGPR) and are well known for colonizing the plant rhizosphere where they suppress soil-borne pathogen at the root surface and stimulate plant growth (Bloemberg and Lugtenberg 2001; Rangarajan *et al.* 2002). Fluorescent *Pseudomonas* is the most promising group of rhizobacteria involved in plant growth promotion and biocontrol of plant diseases (Kloepper et al. 1992).

γ-subclass of Proteobacteria includes mostly fluorescent *Pseudomonas* as well as few non-fluorescent species. It includes non-phytopathogenic, non-necrogenic strains, *P. fluorescens*, *P. putida*, *P. chlororaphis*, *P. aureofaciens,* and *P. aeruginosa* type species (Palleroni 1993). Biochemical characterization had been employed to identify bacteria based on pigmentation, colony morphology, and reaction to dyes and nutritional requirements. It was observed that *P. fluorescens* and *P. putida* were heterogeneous, wherein *P. putida* was subdivided into biotypes A and B, which became biovars A and B. Likewise, *P. fluorescens* was also subdivided into seven biotypes. Biotypes A, B, C, D, E, and F were then called biovars (bv. I to V) and biotypes D and E became *P. chlororaphis* and *P. aureofaciens* respectively (Palleroni et al. 1984; Bossis et al. 2000), which were then identified as *P. chlororaphis* (Johnson and Palleroni 1989). All the fluorescent *Pseudomonas* fall into one of five rRNA (Palleroni et al. 1973). The G+C (Guanine + cytosine) content of their DNA ranges from 58 to 68-mole percentage (Palleroni 1993).

Pseudomonas fluorescent has been recognized as beneficial to plant growth (Kloepper et al. 1988). Weller (1988) reported that fluorescent *Pseudomonas* is the most exploited bacteria for biological control of diseases. Rhizosphere associated bacteria possess many characters that make them well suited for plant growth-promoting agent. Plant growth is directly enhanced by fixation of Nitrogen, solubilization of phosphate, iron chelation, and phytohormone production and indirectly (by suppressing plant pathogenic organisms, by inducing resistance in the host against biotic and abiotic stress). Lemanceau (1992) introduction of fluorescent *Pseudomonas* strains helps in maintaining the microbial population over the rhizosphere and also brings changes in the bio-physical pathway in inoculated plants. Along with PGPR activity fluorescent *Pseudomonas* also reduces the microbial population of plan pathogenic organisms. The application of potential strains of fluorescent *Pseudomonas* increases soil health and promotes beneficial bacterial activities. Lemanceau et al. (1995) estimated the colonization of fluorescent *Pseudomonas* in two plant species flax (*Linum usitatissiinum* L.) and tomato (*Lycopersicon esculantum* Mill) and assessed the suppression of the soil-borne disease. They isolated and identified *Pseudomonas* from different soil categories like uncultivated soil from the rhizosphere, rhizoplane, and root tissue of flax and tomato using various classical, biochemical, and physiological tests. The clustering of isolates shows a higher level (87.8%) of similarity. *Pseudomonas* isolates from uncultivated soil and flax plant rhizosphere and concluded that there is a difference in species of *Pseudomonas*. Similarly, comparison of *Pseudomonas* biovar was done among the isolates derived from the rhizosphere of flax and tomato plant, using different biochemical assay isolates had been characterized as *P. fluorescens* biovar II, III, and V and *P. putida* biovar A and intermediate type *P. putida* biovar A has been isolated from root tissue of flax and *P. fluorescens* bv. II has been isolated from the tomato plant. Using repetitive extragenic palindromic PCR fingerprinting phenotypic and genotypic characterization of isolates was done. However, they emerged as potentially most promising group bacteria involved in plant growth promotion and biocontrol of plant diseases (Preston 2004).

2.1.1 Isolation and identification of fluorescent *Pseudomonas* strains

Bacterial colonies of fluorescent *Pseudomonas* species on Kings’B medium can be identified using UV light illumination by its fluorescens. Strain identification can be done by phenotypic analysis (Palleroni et al. 1984). Misko and Germida (2002) isolated bacteria (n=2257) from the rhizosphere and root interior of six cultivars of field-grown canola and identified by fatty acid methyl ester

(FAME) analysis, and confirmed 35% were identified as *Pseudomonas putida* and *P. chlororaphis*. Results implicated that use of *Pseudomonas* as biofertilizers, biocontrol agents, and plant growth-promoting bacteria in canola. Kaiser et al. (2001) isolated 111 microbial communities from the rhizoplane of oilseed rape (*Brassica napus*) and cultivated on different media to establish a culture collection and 16S rDNA sequence analysis revealed that more than 64% of cultivated isolates were allocated to γ-subclasses of proteobacteria. Reddy et al. (2007) isolated and evaluated antifungal activity of 30 *P. fluorescens* isolates against *M. grisea, R. solani, S. oryzea* that are known to attack rice plant. Ramezanpour et al. (2011) isolated 111 strains of fluorescent *Pseudomonas* and evaluated for IAA, siderophore production, and phosphate solubilization. All the isolates have growth promotion activities for the development of rice and its crop yield. 16S rDNA gene sequencing confirmed that these bacteria belong to *P. fluorescens, P. aeruginosa,* and *P. putida.*

2.1.2 Biochemical and molecular characterization of *Pseudomonas* strains

Most of the tests conducted for the identification of bacteria have been based on physiological and nutritional tests (Krieg and Holt 1984). Among the *Pseudomonas* group, *P. aeruginosa* grows above 41^{o}C (Hildebrand et al. 1992). Most of the associated *Pseudomonas* spp. belongs to *P. aeruginosa*. However, *Pseudomonas* species were identified based on the utilization of different carbohydrates (Liu 1952). Sandiford (1937) studied that *P. fluorescens* utilized glucose, mannitol, sucrose, and lactose and formed acid from glucose but *P. aeruginosa* did not form acid from glucose. Blazevic et al. (1973) studied biochemical characterization and antibiotic sensitivity of fluorescein producing strain of *Pseudomonas* by rapid tests for nitrate reductase, gelatin test, and antibiotic sensitivity test for differentiating the strain of fluorescent strains (*P. fluorescens, P. putida, P. aeruginosa*). Bauer (1966) reported a paper disk method for determining resistance/susceptibility of bacteria to antibiotics and chemotherapeutic agents.

Stanier et al. (1966) studied the primary differentiation of species and species group of nearly 267 individual strains and assigned them to one of 10 species. Taxonomically most complexes of these were the fluorescent group in which they recognized three species *Pseudomonas aeruginosa*, *Pseudomonas fluorescens,* and *Pseudomonas putida* showed a high degree of phenotypic uniformity. Woese (1987) on basis of analysis of 16S rRNA, documented REP like sequences in 7 of the 10 phyla by using PCR and DNA-DNA hybridization. Lupski and Weinstock (1992) mini reviewed short, interspersed repetitive genomic DNA sequences in prokaryotic.

Sharples and Lloyd (1990) reported highly conserved repetitive DNA sequences within the intergenic regions of *E. coli, Solmonella typhimurium* chromosome, and were capable of forming stem-loop structure. Dutrecq et al. (1991) identified 2,4 diacetylphloroglucnol in culture filtrate of a strain of *Pseudomonas* fluorescens with antagonist effect on the take-all disease of wheat by mass spectrometry. Strains of *Pseudomonas aeruginosa* were recognized by the production of pyocyanin pigment in addition to their colony character and grape-like savor of amioacetophenone (Gilardi 1991)

Fenton *et al.* (1992) synthesized 2.4 diacetlyphloroglucinol (Phl) from the F113 strain of fluorescens *Pseudomonas* using Tn5 mutagenesis. Seed bacterization with F113 strain of fluorescens *Pseudomonas* in sugar beet enhanced seed germination significantly greater than seed inoculated with F113G22 (Phl producing negative mutant). Hassan and Fridovich (1980) reported pyocyanine is toxic to a wide range of soil-borne fungi. Ten isolates were characterized by five tests: gelatinase, starch hydrolysis, nitrate reduction, antibiotic test, and growth at 4^{o}C and 42^{o}C. All variables were tested by the methods described by Stanier *et al.* (1966). Keel *et al.* (1996) studied 2, 4 diacetylphloroglucinol (Phl) a broad-spectrum antibiotic that determines the biocontrol ability of *Pseudomonas fluorescens* against a wide range of plant pathogens. *P. fluorescens* Q2-87, carrying Phl biosynthesis genes, which was used as a probe for determination of Phl biosynthesis locus within Phl producing *Pseudomonas* strains of worldwide origin.

Bossis et al. (2000) described phenotypic and genotypic characters, the status of taxonomy, and the ecology of *P. fluorescens* and *P. putida*. Genotypic characterization was based on the variability of the consensus gene (rDNA) or of the total genome (DNA-DNA hybridization). Botelho (2001) summarized and discussed significant aspects of rhizosphere microbes and studied taxonomic, genetic, and ecological aspects of fluorescent *Pseudomonas* in the rhizosphere. Proudy *et al.*(2008) performed BOX-PCR fingerprinting for typing of *Ent. Sakazakii* strains in a faster and easier way when compared to the PAGE technique. Waturangi *et al.* (2012) studies showed that ERIC sequences were more informative than REP sequence for analysis of *V. cholera* diversity.

Yoke-Kqueen *et al.* (2013) suggested BOX-PCR might be an effective fingerprinting tool over ERIC and RAPD to determine the origin and divergence of virulent *Vibrio parahaemolytic* strains found in local seafood. Kumar et al (2014) isolated 22 *Pseudomonas* isolates and 17 isolates were biochemically characterized. Isolates Ps-AB4, Ps-JN-2, and Ps-BK-1 showed higher auxin production ranging from 79.67-110.12 µg/L and also produced ammonia, siderophore, and phosphate solubilization. *Pseudomonas* isolates Ps-AB4, Ps-JN-2, and Ps-BK-1may be used as inoculants biofertilizers for wheat cultivation.

Trivedi *et al.* (2015) Based on nucleotides homology and phylogenetic analysis using 16S rDNA sequencing, the isolated sample was detected to be *Pseudomonas entomophila* (Accession Number: AY907566) with 96% identity of gene sequencing data, which was nearest homolog species to *P. fluorescens* (Accession No. EF672049). Biochemical characterization was done to distinguish the bacterial strains and among four isolated bacterial strains, B-2 was identified as *Pseudomonas aeruginosa* (Su, Lae and Ngwe, 2018).

2.1.3 Carbohydrate utilization by fluorescent *Pseudomonas*

Bacteria of different genera (*Pseudomonas, Alcaligenes, Bordetella, Agrobacterium, Moraxella, Acinetobacter, Flavobacterium,* and some CDC groups) were explored for the ability to utilize forty-two carbon substrates with the automated standardized micro method. Many species showed a different pattern of carbon utilization and hence they can be differentiated from each other within genera based on the utilization of carbon especially with representatives of the families *Pseudomonadaceae* and *Alcaligenaceae* (Kämpfer *et al.*, 1988).

P. aeruginosa has a definite biochemical pattern reported by Liu (1952), utilize glucose, mannitol, terhalose, and glycerol as the sole source of carbon for growth and produce acid from glucose. Hugh and Leifson (1953) studied the basic difference between oxidative and fermentative metabolism of carbohydrate. The method presented which serves to distinguish between the two metabolic patterns and differential utilization of carbohydrates for taxonomic characterization of *Pseudomonads*. Agrawal et al. (2015) studied carbohydrate utilization of 24 *P. putida* isolates as identified by the Hi-carbohydrate kit test. All the isolate utilized xylose, esculin, and citrate but exhibited a varying degree of carbohydrate utilization towards other sources of carbon fructose, terhalose, L-arabinose, and malonate. The numerical analysis revealed that all the *P. putida* isolates were polymorphic.

2.2 Fluorescent *Pseudomonas* as plant growth-promoting rhizobacteria (PGPR).

Rhizosphere inhabiting fluorescent *Pseudomonas* is one of the most dominant and potentially most promising groups of PGPR involved in the biocontrol of plant disease (Haas and Defago 2005; Glick 2004). *Pseudomonas* spp. produces an arsenal of antimicrobials and also maintains soil health by employing a wide variety of mechanisms that positively impact plant health. They also can promote plant growth and induce systemic resistance (ISR) in the plant (Glick, 2014).

2.2.1 Solubilisation of phosphate by fluorescent *Pseudomonas*

Phosphate solubilization bacteria are common in the plant rhizosphere. Microbes convert the insoluble form of phosphorous to soluble form and make it available to plants (Kim et al. 1997). Sultenfuss and Doyle (1999) studied that phosphorous is one of the 17 nutrients essential for plant growth. An adequate supply of phosphorous is required for optimum growth, reproduction, and required by crops in large amounts for crop production. Total P concentration in agriculture crops varies from 0.1 to 0.5%. Khan et al. (2009) used conventional methods for metal detoxification of soil. In metal contaminated soils, PGPR maintains soil fertility. Besides the role in metal detoxification, PGPR also plays a major role in plant growth by other mechanisms like phosphate solubilization.

Glick (2012) Soil regardless of a large reservoir of phosphorous, the quantity of phosphorous available to plants is very low because the majority of soil phosphorous is found to be in unavailable forms or present as apatite or as inositol phosphate (soil phytate). Plants absorb phosphorous only in two soluble forms, monobasic ($H_2PO^-_4$) and dibasic ($H_2PO^{2-}_4$) ions (Bhattacharyya and Jha 2012). Fluorescent *Pseudomonas* species such as *P. putida, P. fluorescens,* and *P. aeruginosa* have been identified as phosphate solubilizing bacteria (Otieno et al. 2015). Paul and Sinha (2017) isolated bacterial strain KUPB12 from river Ganga, found to be an effective phosphate solubilizing agent with index 2.85 in pikovskaya's agar plate along with very high soluble phosphate production of 219.64 ±0.33 µg/ml in the liquid medium. 16S rRNA gene sequencing identified the strain as *Pseudomonas aeruginosa*.

2.2.2 Production of IAA (indole 3-acetic acid)

Patten and Glick (1996) IAA is a phytohormone that influences various cellular functions and regulates plant growth. Besides, microbes synthesize IAA with a mechanism to influence plant growth. Khare & Arora (2010) studied that isolate TO3 (*P. Aeruginosa*) selected from 103 isolates showed the highest IAA production and it showed antagonistic activity against *M. phaseolina* and also produced siderophore and HCN. It was screened for the role of bacterial IAA in inhibiting *M. phaseolina* occurring in chickpea. IAA-defective mutant TO52 showed a decrease in the efficacy and promotion of biocontrol as compared with wild isolate TO3. This study revealed the involvement of IAA in plant disease suppression in synergism with siderophore and HCN.

Suzuki et al. (2003) studied IAA biosynthesis in strain HP72 (*P. fluorescens*) and characterized for production of 2, 4-diacetylphloroglucinol, HCN, siderophore, and IAA production. *Pseudomonas* strain HP72 colonized on bent grassroots and induced root growth reduction. IAA production by strain HP72 may play a major role in the construction of a short root system and colonize roots.

Farzana and Radizah (2005) studied that IAA producing fluorescens *Pseudomonas* has a stimulatory effect on the growth of the plant. In sweet potato cultivar, IAA producing fluorescens *Pseudomonas* increased the plant growth by the N, P, K, Ca, and Mg uptake. Karnwal (2009) tested *P. fluorescens* and *P. aeruginosa* for their ability to produce IAA in pure culture in the absence and presence of precursor L-tryptophan at concentration 50, 100, 200, and 500µg/ml.

Indole concentration in both strains increased with an increase in the concentration of L-tryptophan. *P. aeruginosa* produced less IAA than *P. fluorescens*.

Spaepen and Vanderleyden (2011) reviewed that bacterial auxin can cause interference with the many plant development processes regulated by auxin. Naturally, occurring auxin acts as a signaling molecule in microorganisms because IAA affects gene expression in some microorganisms. IAA acts as a reciprocal signaling molecule in microorganisms in microbe-plant interaction.

2.2.3 1-aminocyclopropane-1-carboxylate (ACC) deaminase

Glick (1995) developed a fast procedure for isolation microbes associated with rhizosphere by assessing the ability to utilize the 1 -aminocyclopropane-1 – carboxylate as the sole source of nitrogen in the presence of enzyme ACC deaminase and *Pseudomonas* isolated from the soil utilized the ACC as a nitrogen source as it is present in the soil bacteria. This method can replace the slow and tedious method of testing each and individual isolates for their growth-promoting activity. Khalid et al. (2006) studied the efficacy of substrate 1-aminocyclopropane-1-carboxylic acid (ACC) (0.1g/kg soil) for ethylene biosynthesis in sandy clay loam soil by gas chromatography and revealed that soil amended with ACC significantly stimulated ethylene biosynthesis in soil.

Saleem *et al.* (2007) studied that ACC deaminase regulates ethylene biosynthesis by metabolizing ACC into α-ketobutyrate and NH_3. Inoculation of microbes containing ACC deaminase activity could help sustain plant growth and development under different stresses. Nadeem et al. (2007) isolated 20 rhizobacterial strains containing ACC deaminase from the rhizosphere of the salt-affected maize field. Among them isolate S20 (*Pseudomonas fluorescens*) were found to be the most effective strains for plant growth promotion and yield in maize even in high salt stress. Magnucka and Pietr (2015) isolated three strains of fluorescent *Pseudomonas* from wheat and rape rhizosphere. Among them, strain RZ310 (*Pseudomonas brassicacearum subsp. Brassicacearum)* showed the highest activity of ACC during 3rd day of growth in DF salt media.

2.2.4 Siderophores production by fluorescent *Pseudomonas*

Kloepper *et al.* (1980) studied the mechanism by which plant growth is promoted by *Pseudomonas putida* (PGPR) A1, BK1, TL3B1, and B10 was studied and found that they produce extracellular microbial siderophore which chelates iron from the environment making it less available for other microflora residing there and in turn enhances the plant growth. Leong (1986) studied enhanced plant growth and biocontrol activity by the beneficial fluorescent *Pseudomonas* and found that it is partially due to siderophore produced by them. Leong (1986) studied that iron-chelating molecules called siderophores produced by beneficial microorganisms suppressed the growth of take-all caused by *G. graminis* and wilt caused by *F. Oxysporium.* Partially purified siderophore does not suppress the disease. Whereas, fully purified siderophore from suppressive beneficial microorganism induces disease supersession.

Bagnasco et al. (1998) studied that siderophore producing *Pseudomonas* strains UP61, UP143, and UP148 isolated from *Lotus corniculatus* showed antagonistic activity against *Rhizoctonia solani* and *Pythium ultimum*. Sayyed et al. (2005) Siderophore producing ability of NCIM 5096 (*Pseudomonas fluorescens*) and NCIM 2847 (*Pseudomonas putida*) was investigated and found that both the strains produce maximum siderophore of hydroxymate type. Both the isolates when inoculated in wheat resulted in increased seed germination, root and shoot length under pot culture condition. Hartmann et al. (2009) suggested that the rhizosphere driven selection of microbes has high potential to improve plant health.

Rajkumar et al. (2010) Metal resistant siderophore-producing bacteria play a major role in the successful survival and growth of the plant and contaminated soil by alleviating the metal toxicity and supplying the plant with nutrient, particularly iron**.** Carvalhais et al. (2013) suggested that Fe-deficient maize exudates induced an ABC transporter for bacterial siderophores in FZB42 (PGPR-*Bacillus amyloliquefaciens*), which may increase Fe availability to plants. Deshwal (2012) studied that maximum siderophore producing *Pseudomonas* strain MR-18 inhibited the growth of *Sclerotina sclerotiorum* by 62-83%. Sajeed &Vidhale (2013) revealed that siderophore is a biological molecule of a low-molecular-weight iron-chelating agent. It is produced by many bacteria of agricultural importance; it enhances the fertility of the soil and acts as a biocontrol agent for the fungal pathogen.

2.2.5 Plant growth-promoting response of fluorescent *Pseudomonas* isolates soybean, chickpea, and wheat following seed bacterization.

Brown (1974) explained about the seed bacterization with *Pseudomonas* enhances the plant growth promotion and yield attributing characters as well as disease control. Due to increased rates of plant growth-promoting activity, many plants like cabbage and lettuce show a huge leaf area that increases the production of photosynthesis assimilates. Also, suppression of disease incidence is recorded in the seedling stage of crops. Bakker and Schippers (1987) discussed that cyanide is a microbial metabolite possibly involved in the decrease of potato yield in potato short-rotation soils. *Pseudomonas* isolates WCS374 and WCS358 producing specific siderophore increase growth by competing with cyanide producing organism for Fe^{+3} in such soils. Kumar and Dube (1992) chickpea and soybean seeds upon seed bacterization of with RBT 13 (fluorescent *Pseudomonas*) resulted in increased seed germination, plant growth, and yield and reduced the number of chickpeas wilted plants in wilt sick soil by 52% making isolate RBT-13 a potential agent against chickpea wilt. Glick (1995) studied ways in which PGPR's facilitate the growth of plants by the indirect and direct mechanism. Frommel et al. (1993) studied the root colonization ability of *Pseudomonas* spp. mutant strains MFE (consistent growth promoter) and IIM15 (inconsistent growth promoter). The root colonization of potato plants was consistently greater with MFE than with IIM15. Seed tuber bacterization with MEF strain stimulated plant emergence, root development in the field, and also increased yields of commercial size tubes (>55mm) when compared to non bacterized control.

Dobbelaere et al. (1999) studied that auxin production by *Azospirillum* is believed to play a major role in the observed plant growth promotion effect. *Azospirillum brasilense* sp245 and sp7 resulted in a strong decrease in root length and increase in root hair formation and the auxin effect was further enhanced by adding tryptophan, a precursor of IAA. Asghar et al. (2002) studied that seed bacterization with potential rhizobacterial strain S54 Significantly increased plant height, stem diameter, number of branches, number of pods per plant, 1000 grain weight, grain yield, and oil content in *Brassica* species over control. O'sullivan and

O'Gara (1992) studied the necessary characters required for the selection or improvement of *Pseudomonas* strains. Production of iron-binding compounds, antibiotic compounds, and efficient root colonization ability has been recognized as important for the inhibition of plant pathogens. Shweta et al. (2008) reported that *Pseudomonas* isolates PS1 and PS2, restricted the growth of *M. phaseolina* by 71% and 74%, respectively by dual culture technique. Both the PS1 and PS2 also enhanced germination in groundnut by 15% and 30% and grain yield by 66% and 77%, respectively over control. Gholami et al. (2009) Seed bacterization with *P. putida* (R-168) in maize enhanced germination and vigor of seed. Seed treatment with R-93 (*P. fluorescens*) significantly increased leaf and shoot dry weight in maize. Seed bacterization with *P. fluorescens* (DSM 50090) showed a stimulating effect on the growth and development of the plant. In maize seed treatment with bioagent enhanced plant height, 100seed weight, shoot dry weight, no. of seeds per ear. Chakraborty et al. (2011) isolated S4 (*Bacillus cereus*) bacteria from the rhizosphere of *Cynodon dactylon* which was found to be highly salt-tolerant, by growing in nutrient agar medium supplemented with 10% NaCl. For the *in-vivo* test S4 was applied as an aqueous suspension to *Vigna radiate, Cicer arietinum,* and *Oryza sativa,* and set of each plant was applied with 200mM NaCl solution. Results revealed that isolate S4 could promote the growth of seedlings significantly which showed increased height, number and length of leaves, as well as root shoot and biomass. Saravanakumar et al. (2011) tested strains of *Pseudomonas fluorescens* (Pf1) and Endophytic *Bacillus subtilis* (EPB-5, EPB-22, EPB-31) for their ability to induce stress related protein and enzymes in green gram. *Pseudomonas* strain Pf1 accumulated higher amount of proline besides, recorded increased vigour index in green gram seedling.

Saber et al. (2012) evaluated the efficiency of PGPR plus nitrogen and phosphorus chemical fertilizer on yield of wheat (cv. N-80-19) in field conditions. Results showed that the application of biofertilizer could increase grain yield and harvest index in wheat as 46.6 and 48.8% compared to control respectively. Malleswari and Bagyanarayana (2013) collected 25 rhizosphere soil samples of aromatic plants and medicinal from different regions of Andhra Pradesh. Four isolates Cf7, Cf60, Te1, and AV 30 were found to be promising PGP activities and were identified as *Pantoea sp, Bacillus sp,* and *Pseudomonas sp* on the basic of colony morphology and Gram staining. All the isolates on seed inoculation enhanced seed germination, root, shoot length, and seedling vigor in sorghum, maize, and green gram. Elekhtyar (2015) conducted two field experiments during 2013 and 2014 in rice cultivar Sakha 105 to study the effect of Rhizobial bacteria in the rice field and its combination with inorganic nitrogen fertilizer on growth. Combination of 46kg N fed^{-1} + 200% of *Rhizobium* fed^{-1} or 46 kg N fed^{-1} + 100% of *Rhizobium* as bio N fertilizer enhanced growth characters in rice cultivar Sakha 105. Using of *Rhizobia* save about 1/3 of inorganic nitrogen fertilizer dose and enhance rice production in sustainable agriculture. Sheirdil et al. (2019) reported the application of root-associated microbes alone or in consortium improves yield in wheat crops. Field studies showed a significant increase in shoot length (T8: *Pseudomonas brassicacearum subs neoauruntiaca*) which gave an 82% increase over control. Maximum growth parameters were recorded in consortium treatment-1 (RA-2, 4, 6) followed by consortium-2 (RA-7, 8, 10) and concluded that the use of microbes in consortium provides a sustainable approach to reduce synthetic fertilizer in wheat production

2.3 Bio-control activity of fluorescent *Pseudomonas*.

Oberhänsli *et al.* (1991) investigated IAA biosynthesis in strain CHA0. Tryptophan transaminase activity was detected in strain CHA0. *Pseudomonas fluorescens* strain CHA0 suppressed take-all disease of wheat or black rot tobacco. Thomashow *et al.* (1990) studied that *Pseudomonas fluorescens* and *P. aeruginosa* produced the antibiotic phenazine-1-carboxylic acid and inhibited the take-all disease of wheat (*Gaeumannomyces graminis var tritici)*. Prashanthi *et al.* (2000) reported strain Pf1 (*Pseudomonas fluorescens*) inhibited *Macrophomina phaseolina* causing safflower root rot. Seed treatment with fluorescent *Pseudomonas* was more effective than soil drenching and the highest survival rates were observed in seed treatment with *P. fluorescens* (86.66%). Delany et al. (2000) studied anti-fungal metabolite 2, 4-diacetyl phloroglucinol plays a major role in the biocontrol capabilities of *P. fluorescens*. Karunanithi *et al.* (2000) recorded pyrollnitrin producing *Pseudomonas* isolates inhibited the growth of *Macrophomina phaseolina*. Weller et al. (2002) studied the nature of microbes in agriculture soil.

They explained the disease suppression by *Pseudomonas* of soil-borne diseases like *Fusarium* wilt, Potato scab, and apple replant and take-all disease. Further suppression of take-all decline disease occurs due to an increase in the inoculums of *Pseudomonas* spp due to the production of antifungal metabolites 2, 4 – diacetyl phloroglucinol. Naturally occurring bacterial strains prevent infectious diseases of plant roost has been analyzed in detail by Haas and Défago (2005).

Weller et al. (2007) explained the disease suppression nature of *Pseudomonas* spp in the take-all disease of wheat caused by *Gaeumanomyces graminis*. The level of antifungal metabolite (2, 4 – diacetyl phloroglucinol) produced by *Pseudomonas* spp to a diversity of 10^5 CFU/g of root acts as a threshold amount to suppress the pathogen. 22 isolates of *Pseudomonas* spp known for 2, 4 – diacetyl phloroglucinol producers have been classified based on the whole-cell repetitive sequence-based REP-PCR analysis. It helped in the development of phylogeny. The presence of many genotypes of *Pseudomonas* species in soil that produces DAGP (2, 4 – diacetyl phloroglucinol) acts as a biocontrol for different plant diseases. Jha and Anjaiah (2007) selected fluorescent *Pseudomonas* strains SAB8 and GM4. The active antifungal metabolites produced by these strains in culture filtrates were tested for growth inhibition of *Fusarium semitectum* and detected inhibition of hyphal tip elongation and an extensive branching of hyphae with cross septa. Saxena (2010) found that *P. aeruginosa* (RM-3) exhibited the innate potential of plant growth promotion and biocontrol potential. Strain RM-3 showed growth inhibition of *Macrophomina phaseolina* in plate assay (68%). Besides, Seed bacterization with strain RM-3 resulted in increased root and shoot length in moong bean.

Kotasthane *et al.* (2017) developed a simple confrontation assay technique for identifying potential bacterial antagonist, it was confirmed that *Pseudomonas* isolates P66 and P141 inhibited the growth of *R. solani* (50.7%) and *S. rolfs* (76%) and in the field experiment, seed bacterization with selected *Pseudomonas* isolates (P66 and P141) resulted in reduced collar rot (*S. rolfsii*) incidence. *Thilagavathi et al.* (2007) tested a combination of *P. fluorescens* + *T. viride* against *M. phaseolina,* resulted in inhibition of mycelial growth of the pathogen.

Senthilkumar et al. (2009) isolated 137 bacterial isolates from soybean root, stem, and nodule tissues and screened for antifungal activity against *Macrophomina phaseolina*, *Fusarium udum*, and *Sclerotium rolfsii* under *in-vitro* plate assay. 16S rDNA analysis revealed that eight isolates belong to the *Bacillus* group and among them a strain of *Bacillus sp*. HKA-121 as potential candidates for charcoal rot biocontrol as well as soybean plant growth promotion. Islam et al. (2018) screened Thirty-five rhizobacterial isolates for antagonistic activity in dual culture, and isolate BA5 (*Pseudomonas aeruginosa*) showed the highest antagonistic activity (58.33% mycelial growth inhibition) against *Fusarium oxysporum* f. sp. *cucumerinum*. Cell-free culture filtrate and ethyl acetate crude extract inhibited mycelial growth of *Fusarium oxysporium* f, sp *cucumerium* by 56.66, and 25.0%, respectively.

2.3.1 *In-vitro* infection model for *Macrophomina phaseolina* causing soybean charcoal rot

Ammon *et al.* (1975) observed that sclerotial germination, hyphal growth pattern, and appressorial formation of the charcoal rot fungus (*Macrophomina phaseolina* (Tassi) Goid) on the surface of soybean roots using SEM. Appressoria are produced on the root surface at the tips of both primary hyphae and side branches within 3DAI. Pearson et al. (1984) demonstrated and monitored root colonization of *Macrophomina phaseolina* causing root rot in soybean at different growth stages and in different cultivars. Bay, Essex, and Forrest (group V cultivars) had the slowest rate of colonization. Sprite (a group III cultivar) had a rate similar to the group V cultivars and yielded like Essex. Fungal population in the root system of soybean at growth stage R8 (harvest maturity) was negatively correlated with yield.

Singh *et al.* (1990) evaluated that chickpea root inoculated with *Macrophomona phaseolina* showed disintegration of cortical tissues and mycelium and sclerotial bodies plugging the xylem were seen in longitudinal section. The sudden collapse of infested plants at the pod filling stage may be due to this plugging of xylem vessels. Pratt (2006) demonstrated a direct observation technique to evaluate animal waste and other agriculture byproducts for bio-control activity against sclerotia of *M. phaseolina*. Survival of sclerotia in soil was more at 50, 75, 100% moisture-holding capacity, less at 0 and 25% moisture-holding capacity, and least at 125% (flooded soil). Incorporation of ground poultry litter eliminated sclerotia. Pande et al. (2010) suggested that crops and pathogens respond differently to altered weather patterns coupled with greenhouse gas emissions. A higher incidence of dry root rot was found in chickpea varieties that are resistant to *Fusarium wilt* between 2000 to 2010 when the temperature exceeded 33^{o}C at the flowering and podding stage. Bressano et al. (2010) described a new method to infect plant with soil borne pathogen which may be applicable to examine the pre-penetration and penetration phase of soil-borne pathogen and also the early response of the host plant by using Hoaglands solution as a growth medium. The developed *in-vitro* method allowed us to obtain roots of soybean plants infected with *M. phaseolina* to characterize the infection process.

Gupta *et al.* (2012) studied *Macrophomina phaseolina* with special reference to charcoal rot of soybean and confirmed that fungus is primarily soil-inhabiting but is also seed-borne in many crops including soybean. The fungus survives in the soil as micro-sclerotia and found that Low C: N ratio in the soil and high bulk density as well as high soil moisture content adversely affect the survival of sclerotia. Pizarro-Tobías et al. (2015) reported events of root colonization by *Pseudomonas putida* which has natural inhabitant of the rhizosphere and exhibits chemotactic response towards plant root exudates and forms biofilms on root surfaces and studied that the genomic machinery necessary for *Pseudomonas putida* to accomplish survival and maintenance in the rhizosphere when a plant-bacteria mutualistic relationship is on-going, is well conserved within the species *P. putida*. Hemmati et al. (2018)) studied histopathology of *M. phaseolina* with two soybean cultivars, Williams (susceptible) and Hadgeston (resistant), and reported infection events in two soybean cultivars. They also reported that in post-penetration steps, there was a significant difference between two cultivars in terms of root colonization by *M. phaseolina* and the disintegration of root tissues.

2.4 Determination of antioxidant activities

As a result of plant-microbe interaction, plants activate molecular mechanisms which refer to the rapid generation of a high amount of reactive oxygen species (ROS). ROS production is common in healthy plants, and they were initially considered just toxic by-products of different metabolic pathways

(Bailey-Serres and Mittler 2006). To overcome ROS toxicity, plants produce enzymatic antioxidants that scavenge oxygen species (Helepciuc et al. 2014). Enzymatic defense mechanism has emerged to protect cells against oxidative injury. The protective system of enzymes for determination of antioxidant and polyphenylprpanoid activities are SOD (Superoxide dismutase) (E.C.1.15.1.1), Peroxidase (POx) (E.C.1.11.1.7) and Lipid Peroxidase (LPO) (Fridovich 1981; Hammerschmidt et al. 1982; Ohkawa et al. 1979) and Phenylalanine ammonia-lyase (PAL) (E.C.4.1.3.5), Polyphenol oxidase (PPO) (E.C.1.14.18.1), total phenolic content (TPC) respectively (Brueske 1980; Gauillard et al. 1993; Zheng and Shetty 2000)

Girotti (1985) studied how the peroxidation of unsaturated lipids takes place and how it can be measured and also discussed several different aspects of free radical-mediated lipid peroxidation. Attention is also given to nonradical, singlet oxygen driven peroxidation, and how it can be resolved from radical driven processes.

Bernards *et al.* (1999) characterized anionic peroxidase associated with suberization response in potato tuber during wound healing. It was 45kD class III (plant secretory) peroxidase that is localized to suberizing tissue and shows a preference for feruloyl substituted substrates that accumulate during wound healing.

Meena *et al.* (2000) reported that foliar application of *Pseudomonas fluorescens* strain Pf1significantly controlled late leaf spot and rust diseases of groundnut under greenhouse condition. Groundnut plants sprayed with pf1 showed an increase in PAL activity and also an increase in phenolic content.

Barceló and Pomar (2001) studied that thioacidolysis of *Z. elegans* lignins reveals the presence of coniferyl aldehyde end groups linked by β-*0*-4 bonds. Both coniferyl and sinapyl alcohols and aldehyde are substrates of a xylem cell wall located strongly basic peroxidase, which is capable of oxidizing them in the absence and the presence of hydrogen peroxide.

Sarma *et al.* (2002) studied the plant growth-promoting efficacy of seven strains *Pseudomonas fluorescens* (pfs 1-7) and were tested for their ability to protect chickpea against *Sclerotium rolfsii* infection under field conditions. Seed treatment of chickpea seeds with *Pseudomonas* strain pfs3 caused maximum accumulation of total phenolic and gallic acid over control or untreated plants. PGPR-mediated induction of phenolic compounds as a biochemical barrier in *C. arietinum* against *S. rolfsii* infection is envisaged. Singh *et al.* (2003) studied that seed treatment with *Pseudomonas fluorescens* strain pf4 and *Pseudomonas aeruginosa* strain pag protected chickpea plants from *Sclerotium rolfsii*. Foliar spray of *Pseudomonas* strains enhanced phenolic acid content as well as total phenolics within 24h of application. Resistance in chickpea plants by *Pseudomonas* strains through the induction of phenolic compounds as well as induced systemic resistance via the SA-dependent pathway was evident.

Yoruk and Marshall (2003) discussed the potential physiological roles of polyphenols (PPO) in the plant. Thipyapong et al. (2004) studied the physiological role of drought-induced PPO activity in tomato.

Passardi *et al.* (2004) reported that peroxidase plays a key role in the plant life cycle, specifically in the modification of cell wall, in roles which can be antagonistic depending on the developmental stage. This diversity of functions derives in part from two possible catalytic cycles of peroxidases involving the consumption or release of H_2O_2 and reactive oxygen species.

Karthikeyan *et al.* (2006) Application of biocontrol agent's *P. fluorescens, T. Viride* in combination with chitin induced accumulation of high levels of phenol and activities of PAL (phenylalanine ammonia lyases), PO (peroxidase), PPO (Polyphenol oxidase), chitinase and β-1,3-glucanase in coconut palm compared to other treatment and control.

Pitzschke *et al.* (2006) studied the mechanism of how to plant sense and respond to ROS are discussed in the context of the biological effects and functions of ROS in the plant. Plants make use of ROS as a signaling molecule for regulating development and various physiological responses. Anand et al. (2007) Treated tomato plants with azoxystrobin and *P. fluorescens* triggered the plant-defense enzymes (PO, PPO, PAL, b-1, 3 glucanases, chitinase, and catalase) and chemical (total phenols) in response to infection by *A. solani* and *S. lycopersici*.

Mandal *et al.* (2008) investigated the intensity and timing of ROS formation, lipid peroxidation, and expression of antioxidant enzymes as an initial response to tomato against invading necrotrophic pathogen *Fusarium oxysporium* f.sp. *lycopersici*.

SOD, CAT, GPX, and APX activities increased in response to pathogen inoculation. It was discussed that oxidative burst served as a weapon for invading necrotic pathogen.

Singh *et al*. (2010) Reported that overproduction of ROS due to abiotic and biotic stresses cause peroxidation of lipid membranes and indicated by the formation of malondialdehyde (MDA). Dai and Mumper (2010) studied a comprehensive overview of phenolic extraction, analysis, and quantification as well as their antioxidant properties. Maurya and Singh (2010) analyzed total phenolic content in *Adhatoda vasica* leaves. TPC varied in the extracts from 63.95 ± 2.1 to 92.4 ± 0.14 mg/g. The phenolic content was found maximum in aqueous extract (92.4 ± 0.14mg/g).

Seo *et al.* (2012) investigated *B. thuringiensis* GS1 isolated from bracken (*Pteridium aquilinum*) to induce resistance to *Rhizoctonia solani* in cucumber plants. SDS PAGE confirmed cucumber plants treated with GS1 induced Guaiacol peroxidase (GPOD), Ascorbate peroxidase (APX) which might have suppressed the damping-off caused by *Rhizoctonia solani* KACC 40111.

Das and Roychoudhury (2014) studied different types of ROS and highlighting the potential role of antioxidants in abiotic stress tolerance and cellular survival. Naqvi *et al.* (2013) investigated for the first time that Pakistan wheat grain was collected from NARC gene banks for their PPO activities. Substrate L-DOPA at pH 6.5 produced the highest enzyme activity. Genotype chalsi showed the highest (150units/min/g) activity of PPO. Suzuki et al. (2014) studied the effect of biotic and abiotic stresses on the plant under controlled conditions or in laboratory conditions.

Singh *et al.* (2014) Reported that the accumulation of SOD, PAL, PPO, when treated with all the beneficial microbes, was enhanced and the maximum increase was seen with the triple microbe consortium confirming their synergistic behavior when applied in the mixture. However, compatible microbial consortium showed the most prominent effect.

Selvaraj and Ambalavanan (2013) studied the induction of defense-related enzymes and phenols accumulation in anthurium by a bio-control agent like *P. fluorescens, Bacillus subtilis,* and *Trichoderma viride.* Application of these bioagents to anthurium induced defense enzymes *viz.*, PAL (phenylalanine ammonia-lyase), PO (peroxidase), PPO (polyphenol oxidase), and peroxidase upon challenge inoculation with *Coletotrichum gloesporioides*.

Islam *et al.* (2014) isolated Zn resistant *Pseudomonas aeruginosa* having an inhibitory concentration of 1500 mg/kg against Zn from arable land irrigated with industrial effluent and evaluated its bioremediation potential. Soil inoculation of Zn resistant *Pseudomonas aeruginosa* to measure MDA accumulation and H_2O_2 production in wheat under Zn stress resulted in increased enzymatic (SOD, POD, and CAT) and nonenzymatic (total phenols and ascorbic acid) as compared to Zn treated plants. P. aeruginosa was used in the wheat crop against Zn induced oxidative stress.

Kumar *et al.* (2016) studied strains of root-associated microbes NBRIRA (*Pseudomonas putida*) and NBRISN13 (*Bacillus amyloliquefaciens*) showed tolerant to abiotic stress when inoculated in a consortium for improving chickpea growth under control and drought-stressed conditions. In the improvement of plant growth, the synergetic application of PGPR acquired a better place to overcome different stresses.

Khan *et al.* (2019) treatment of chickpea plants with a consortium of PGPR and PGRs considerably enhanced the leaf proline content, lipid peroxidation and activities of antioxidant enzymes (CAT, APOX, POD, AND SOD) all increased in response to drought stress but decreased due to the PGPR and PGRS treatment.

Khetarpaul (2018) studied phenolic content (0.87 to 0.95 mg GAE/100g), DPPH scavenging activity (31.9 to 32.8%), and flavonoid content in sags containing 20% fresh chickpea leaves at 45 DAS of desi and Kabuli chickpea varieties (HC-1, C-235, HK-1, and HK-2).

CHAPTER-III
MATERIALS AND METHODS

The present investigation entitled "**Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters and induction of antioxidant enzymes in soybean, chickpea, and wheat"** was carried out in the Department of Plant Pathology and Department of Plant Molecular Biology and Biotechnology, College of Agriculture I. G. K. V, Raipur to isolate and characterize fluorescent *Pseudomonas*. Field and pot experiments were carried out at research cum instructional farm, College of Agriculture, I. G. K. V, Raipur. The materials used and methodology adopted for the study is described in this chapter.

3.1 Materials

3.1.1 Fluorescent *Pseudomonas* isolates

Fluorescent *Pseudomonas* isolates used in the present study were isolated from different places of Bastar and 4 isolates of fluorescent *Pseudomonas* were isolated from *Brassica* specific root rhizosphere. The isolates were used for biochemical and molecular characterization and screened for siderophore production, ACC deaminase production, IAA production, phosphate solubilization, and antagonistic activity. The potential fluorescent *Pseudomonas* isolates were then tested for growth and yield attributing parameters following seed treatment in pot and field experiments.

3.1.2 Plant material

The seed of soybean (CG SOYA-1), chickpea (INDIRA CHANA-1), and wheat (Amber wheat) used for pot and field experiments were obtained from KVK farm, College of Agriculture, I. G. K. V, Raipur.

3.2 Methods

3.2.1 Collection and isolation of fluorescent *Pseudomonas*

Approximately 88 soil samples collected from different locations of Bastar, Bilaspur, and 4 soil samples collected from *Brassica* specific root rhizosphere from fields of Telangana (Zhahirabad) were used for the isolation of fluorescent *Pseudomonas.* Isolation was done by standard serial dilution method on Kings's B media agar plates. The plates were incubated at 28^{o}C for 48 hours. Fluorescent *Pseudomonas* single bacterial colonies were identified under the UV light (366nm) and further purified by streaking on to the Kings'B media plates. Single pure culture colonies of these isolates were subcultured and maintained on King's B media slants.

3.2.2 Biochemical characterization of fluorescent *Pseudomonas* isolates

Various biochemical tests were used for the characterization of potential fluorescent *Pseudomonas* isolates. The composition of different media used for the tests is summarized in Table: 3.9

3.2.2.1 Starch hydrolysis test

The starch hydrolysis test is used to detect the enzyme amylase, which breaks down starch. Amylase hydrolyses starch, a polysaccharide into maltose, a disaccharide, and some monosaccharide such as glucose. Isolates were single streaked on starch agar media and incubated at 28 ± 2^{o}C for 48 hrs. After the required incubation, a few drops of Lugol's solution were added. A yellow zone around the colony indicated starch hydrolysis, while a blue/black area around them indicated the presence of starch.

3.2.2.2 Gelatin liquefaction test

The gelatin hydrolysis test is used for identifying and differentiating species of *Pseudomonas*. Gelatin is a protein that dissolves in warm water and exists as a liquid above 25^{o}C and solidifies when cooled below 25^{o}C. Gelatin hydrolysis was detected by observing liquefactions at low temperature (4^{o}C) after incubation of the *Pseudomonas* isolates in it at 28^{o}C for 48 hrs. Gelatin inoculated tubes that remain liquefied produced gelatinase and showed a positive test for gelatin hydrolysis and tubes that remained solid were negative.

3.2.2.3 Growth at 4^{o}C and 42^{o}C

Pseudomonas isolates were inoculated in King's B medium, incubated at 4^{o}C for 48 hrs and observation was taken. The growth of the organism at 4^{o}C indicated positive results. *Pseudomonas* isolates were inoculated in King's B medium, incubated at 42^{o}C for 48hrs and observation was taken. The growth of the organism at 42^{o}C indicated positive results.

3.2.2.4 Antibiotic sensitivity test

An antibiotic sensitivity test was used to distinguish different species of fluorescent *Pseudomonas* (*P. fluorescent/P. putida/P. aeruginosa*). The streak plate method of Bauer (1966) was used to perform an antibiotic sensitivity test. Kanamycin and carbenicillin sensitivity was determined by incorporating 1mg/ml of kanamycin and 0.1mg/ml of carbenicillin, respectively, in King's B medium. *Pseudomonas* spp. showing positive growth on either of the antibiotic supplemented medium was resistant.

3.2.2.5 Carbohydrate utilization test

For testing the carbohydrate utilization profiles of fluorescent *Pseudomonas* isolates Hicarbohydrate^{TM} kit (Himedia Laboratories, Mumbai, India) were used. Fresh bacterial cells were grown in King's B broth and used for inoculation. 50 µl of an aliquot of the fresh cell suspension was inoculated to each well in the kit. On incubation of the kit at 28^{o}C for 24-48hrs bacterial cells undergo metabolic changes which are indicated by a spontaneous color change in the media. The results were recorded as per the manufacturer's instructions.

3.2.2.6 Screening isolates of fluorescent *Pseudomonas* for phosphate solubilization

3.2.2.6.1 Qualitative estimation

Qualitative screening of phosphate solubilizing fluorescent *Pseudomonas* was performed on Pikovskaya's agar medium (Himedia) containing tricalcium phosphate as a phosphate source and Bromo cresol purple (0.1 g/l) as a pH indicator for acidification (Vazquez et al. 2000). Sterile Pikovskaya's agar medium was poured into fresh plates and the actively growing fresh culture of *Pseudomonas* isolates was stab inoculated in media. After incubation of inoculated plates at 28±2^{o}C for 2 days, the phosphate solubilizing isolates turned the media color from purple to yellow in the zone of acidification.

3.2.2.6.2 Quantitative screening in Pikovskya's medium

Quantitative screening of phosphate solubilization in Pikovskya's broth was performed according to the procedure of Murphy and Riley (1962). Actively growing fresh cultures of potential *Pseudomonas* isolates were inoculated in Pikovskya's broth to evaluate its ability to solubilize supplemented phosphate. 50 ml of Pikovskya's broth was poured into a 100 ml conical flask and autoclaved. After sterilizing Pikovskya's broth was cooled and inoculated with *Pseudomonas* cultures and incubated at 28±2^{o}C 100rpm. After seven days of incubation, the amount of inorganic phosphate (Pi) released in the broth was estimated in comparison with uninoculated control. Then broth culture in the flask was transferred to 50 ml tarson tubes and centrifuged at 10000 rpm for 10 minutes. It separates the supernatant from bacterial growth and insoluble phosphate. 5ml of chloromolybdic acid (CMA) was added to 0.5 ml of culture supernatant and mixed thoroughly. The color change after 15 minutes was read in colorimeter at 610nm. Blank was prepared with DW, reference or control was prepared with un-inoculated broth supernatant instead of sample supernatant. Phosphate solubilization activity was expressed in terms of tri-calcium phosphate solubilization which in turn was measured by µg/ml of available orthophosphate as calibrated from the standard curve of potassium dihydrogen phosphate.

The standard solution was prepared with KH_2PO_4 (10mg/ml) in distilled water with the increasing concentration of stock 0.0µl, 1µl, 2.5µl, 5µl, 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, 70µl, 80µl, 90µl, and the concentration of KH_2PO_4 (10mg/ml) was 0.0µg, 10µg, 25µg, 50µg, 100µg, 200µg, 300µg, 400µg, 500µg, 600µg, 700µg, 800µg, 900µg. Measurement of optical density (OD) was taken at 610nm.

3.2.2.7 Determination of Indole Acetic acid (IAA) production

For the production of an indolic compound, an actively growing fresh culture of fluorescent *Pseudomonas* was inoculated to 20 ml of DF salts minimal media (Dworkin and Foster 1958) in 100 ml conical flask and incubated for 4 days at 28±2^{o}C. The medium was supplemented with L-Tryptophan at a concentration of 1.02g/L from a 5mM filter-sterilized stock prepared in warm distilled water. After incubation for 72hrs, the grown bacterial cells were removed from the culture medium by centrifugation at 5000 rpm for 5 minutes and the pH of the medium of all isolates was recorded. Then 1ml of an aliquot from the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Weber 1951) with blank as uninoculated DF salts minimal media. It was allowed to stand at room temperature for 20 min before the absorbance at 535 nm was measured in the colorimeter.

The standard curve was prepared by adding 1M IAA in increasing concentration 0.0µl, 5µl, 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, 70µl, 80µl, 90µl, 100µl in DF salt minimal media with decreasing concentration: 1ml, 995µl, 990µl, 980µl, 970µl, 960µl, 950µl, 940µl, 930µl, 920µl, 910µl, 900µl respectively. Then 4 ml of Salkowski's reagent was mixed vigorously in each tube followed by incubation at room temperature for 20 minutes after which absorbance at 535 nm was measured in the colorimeter. The concentration of IAA in each culture was determined by comparison with the standard curve.

3.2.2.8 Screening of 1-aminocyclopropane-1-carboxylic acid deaminase (ACC Deaminase) containing fluorescent *Pseudomonas* isolates.

The availability of 1-aminocyclopropane-1-carboxylic acid deaminase (ACC Deaminase) as a nitrogen source is because of the enzymatic activity of ACC deaminase. The material required and the protocol for utilization of ACC are as follows.

1. DF salt minimal media
2. Trypticase soya borth
3. 0.1M Tris HCL (pH 7.5)
4. 0.1M Tris HCL (pH 8.0)
5. 0.1M Tris HCL (pH 8.5)
6. 0.5M solution of ACC
7. $(NH_4)_2SO_4$ (0.2% w/v)
8. 5%toluene (v/v)
9. 0.56N HCL
10. DNF solution (0.1g 2,4-dinitrophenihydrazine in 100 ml of 2N HCL)
11. 2N NaOH
12. α-ketobutyrate
13. 0.1N NaOH
14. Bradford's reagent
15. Bovine serum albumin (BSA)

Preparation of ACC:

0.5M solution of ACC (sigma-Aldrich Co., Mumbai, India), which is labial in solution, was filter sterilized through a 0.2mm membrane and the filtrate collected aliquoted and frozen at -20°C. Just before inoculation, the ACC solution was thawed.

3.2.2.8.1 Qualitative estimation of ACC Deaminase activity

1. *Pseudomonas* isolates were grown in 5ml of TSB medium and incubated at 28°C at 120 rpm for 24 hrs.
2. The cells were harvested by centrifugation at 3000 rpm for 5 minutes and washed twice with sterile 0.1M Tris-HCl (pH7.5) and re-suspended in 1 ml of 0.1M Tris-HCL (pH 7.5) and spot inoculated on Petri plates containing modified DF (Dworkin and Foster) salt with minimal medium (Dworkin and Foster, 1958) supplemented with 2mM ACC as a sole nitrogen source
3. Plates containing only DF salt minimal medium without ACC as negative control and with $(NH_4)_2SO_4$ (0.2%w/v) as a positive control.
4. Plates were incubated at 28°C for 27hrs
5. The growth of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as a nitrogen source.

3.2.2.9 Screening and characterization of potential *Pseudomonas* isolates for siderophore production

3.2.2.9.1 Hydroxyquinoline mediated siderophore test

Pseudomonas isolates that have a high ability to produce siderophore were identified by inoculating the isolates on King'sB medium supplemented with 8-Hydroxyquinoline (50mg/l) which is a strong chelator. After supplementation with this strong chelator, it reduces the availability of iron in the medium. Isolates that can produce siderophore can grow on this medium. Inoculated isolates were then incubated at 28±2°C for 48-72 hrs.

3.2.2.9.2 Quantitative assay for siderophore production (liquid assay)

The chrome azurol sulfonate (CAS) assay (Schwyn and Neilands 1987) was used to evaluate the siderophore production. Actively growing cultures of potential fluorescent *Pseudomonas* was inoculated to 20ml of King's B broth in a flask of 100 ml capacity and incubated for 72 hrs at 28±2^{o}C. Centrifugation was carried out at 3000 rpm for 5 minutes to sediment the bacterial cells. Pipette out 0.5ml of supernatant and then mixed with 0.5 ml of CAS solution and then add 10µl of shuttling solution (sulfosalicylic acid). After incubation for 20 minutes the color formed was determined by using a spectrophotometer at 630 nm. King's B broth was used as a blank and a reference solution was made by adding CAS dye and shutting solution to King's B broth. Values of siderophore released in King's B medium was expressed in percent siderophore units and calculated by using the formula given below:

$$\textbf{Percent siderophore unit} = \frac{\textbf{(Ar-As)}}{\textbf{Ar}} \textbf{ x 100}$$

Where: Ar = Absorbance or OD of reference solution
As = Absorbance or OD of samples

3.2.3 *In-vitro* screening for antagonistic activity by fluorescent *Pseudomonas* against *Macrophomina phaseolina*

Fluorescent *Pseudomonas* isolates were tested for their efficacy as a biocontrol agent against *M. phaseolina* causing charcoal rot in soybean. Fluorescent *Pseudomonas* isolates were multiplied on King's B broth and incubated for 2 days at 28^{o}C till fluorescent pigment appeared in the broth. Petri plates containing pre-sterilized potato dextrose agar (PDA) medium were inoculated with fungi causing charcoal rot of soybean (*M. phaseolina*) and incubated at 28±2^{o}C for 3 days till fungus completely covered the entire plate. Bipartite interaction was performed following a simple confrontation assay developed by Kotasthane et al. (2017) where edges of a glass funnel were deployed for bio-agent inoculum deposition surrounding pre-inoculated fungal pathogen *M. phaseolina* on the mixer of potato dextrose agar (PDA) and King's B media in equal volume. The edges of the glass funnel were sterilized by dipping in alcohol followed by flames. Broth containing young growing cell of fluorescent *Pseudomonas* was dispensed in a sterile petri dish and picked at the edge of the funnel by dipping. Care was taken to remove the excess inoculum by gently shaking the funnel. Inoculation was done by gently touching the edge of the funnel containing inoculum of *Pseudomonas* which encircles the pre-inoculated plant pathogenic fungi on agar equidistantly. The inhibition zone was measured after 72hrs of incubation at 28±2^{o}C. Percent inhibition of pathogen by fluorescent *Pseudomonas* over control was calculated using the formula of (Vincent 1947).

$$\textbf{Percent Inhibition} = \frac{(C-T)}{T} \times 100$$

Where, C=Growth of pathogen in control
T= Growth of pathogen in presence of *Pseudomonas* isolates

3.2.4 Mass multiplication *Macrophomina phaseolina*

Macrophomina phaseolina (*Mp*) obtained from field infected soybean plant, was grown on PDA plates at 28° C. The inoculum of *M. phaseolina* was prepared and mass multiplied on sorghum grains. The inoculation method was adapted from Mengistu et al., (2007). Sorghum (*Sorghum bicolor* L.) grains were used for mass multiplication of *M. phaseolina,* sorghum grains soaked in tap water for 12 hr before autoclaving. Overnight soaked grains were filled in a 500 ml flask (250 gm/flask) and autoclaved at 121° C at 15lbs pressure for 15 min. Flasks containing autoclaved sorghum grains are inoculated with 3 days old culture of *M. phaseolina* and incubated at 25 ± 2°C for 15 days. The flasks were shaken periodically so that uniform colonization of sorghum grains with fungus occurs and grains get darkened with microsclerotia.

3.2.5 Studies on bipartite and tripartite interaction using potential fluorescent *Pseudomonas, Macrophomina phaseolina,* and soybean model system.

Bipartite interaction (interaction between soybean roots x *M. phaseolina*) and tripartite interaction (interaction between soybean roots x *M. phaseolina* x fluorescent *Pseudomonas*) will be done using the proposed soybean model system. In the soybean model system plastic Petri plates (90mm) will be used, slightly modified by cutting one edge in an arch. Soybean seeds treated with *Pseudomonas* isolates will be used for tripartite interaction. Petri plates with soil sowed with soybean seeds and maintained in a growth chamber provided with fluorescent light (12 hr light/12hr dark regime). After good root development, roots will be inoculated with microsclerotia of *M. phaseolina* for studying bipartite interaction. The infection moment was monitored with stereo binocular microscopic by slowly removing the Petri plate lid. Infected root samples were collected for further analysis.

3.2.6 Effect of seed bacterization with *Pseudomonas* isolates on the germination and growth of soybean seedlings.

The potential fluorescent *Pseudomonas* isolates were grown in 100 ml Kings B base broth in 250ml conical flasks on a rotary shaker (150 rpm/min) for 48hrs at room temperature. The culture broth was used as bacterial inoculum. The bacterial suspensions were prepared and tested for their plant growth-promoting activity following seed bacterization. Soybean seeds (CG SOYA-1) were used for testing the efficacy of potential fluorescent *Pseudomonas*. The slurry for seed bacterization was prepared by adding bacterial culture and talcum powder to the seeds of the soybean. Care was taken for uniform coating of all seeds, which were blot dried, placed in wet blotters, and two-third of the blotter was immersed in water and incubated in a growth chamber for 7 days. Untreated seeds untreated were kept as control. Observations were recorded on the 7th day. Ten seedlings were taken randomly from each bacterial treatment and their shoot length and root length were recorded.

3.2.7 Pot experiments

Soybean (CG SOYA-1) and chickpea (INDIRA CHANA-1) seeds were used for the pot experiment. Seed bacterization was done as described in the above paragraph. Seeds treated with potential fluorescent *Pseudomonas* strains were sown at 10 seeds per pot separately. Untreated control pots were maintained for comparative studies. Three replicas were maintained for each treatment and observation such as shoot length, root length, root volume, number of branches, number of pods, etc were recorded during the maximum maturity stage of the crop.

3.2.8 Assessment of growth and yield attributing characters of potential fluorescent *Pseudomonas* isolates in soybean, chickpea, and wheat.

Field experiments were conducted at Research cum instructional Farm, College of Agriculture, I. G. K. V, Raipur, to study the efficacy of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters following seed treatment in soybean (CG SOYA-1), chickpea (INDIRA CHANA-1) and wheat (AMBER WHEAT). Seed treatment was done by spraying potential *Pseudomonas* isolates on seeds and talcum powder was sprinkled so that bacterial culture could coat the seeds completely and shade dried. Seed treatment was done a day before sowing. Three replications were maintained for each treatment in randomized block design and untreated control was maintained for comparative purpose. Observation such as plant height, root length, root volume, number of branches, grain yield, straw yield, biological yield, etc was recorded.

3.2.9 Antioxidant enzyme assay

Different enzymes were assayed in leaves of chickpea and wheat. Seeds were treated with different strains of fluorescent *Pseudomonas* during the crop maturity stage. For the preparation of the leaf sample, fresh green flag leaves of chickpea and wheat were collected from the field and taken to the laboratory in fresh form by keeping the samples in an icebox. Leaf samples were frozen in liquid nitrogen in the laboratory and ground to a fine powder using a fresh pestle and motor and powder was collected in a 50 ml tarson tube and stored at -20^{o}C for further analysis.

3.2.9.1 Superoxide dismutase activity (SOD) (E.C.1.15.1.1)

SOD activity was measured by the method described by Fridovich (1981) using the riboflavin/methionine system.

Principle

Superoxide radical reduces the NBT resulting in the formation of blue color formazan. NBT method for SOD assay is based on the principle that NBT undergoes photoreduction (which is a blue-colored formazan) on exposure to light by superoxide radicals. It competes with enzyme SOD for superoxide anions. In presence of SOD in the reaction mixture, NBT will produce less amount of colored complex than control (therefore less OD than reference or control without enzyme).

Solution

1. 0.1M Phosphate buffer containing 0.5 mM E.D.T.A
2. 100mM Phosphate buffer
3. 200 mM methionine
4. 2.25 mM NBT
5. 1.5 M Sodium carbonate
6. 2µM riboflavin

NOTE: Mix 2,3,4,5 solutions in a beaker to form a reaction mixture

Procedure

1. 0.1 Gram of leaf sample was homogenized with a pre-chilled 0.1M Phosphate buffer containing 0.5 mM E.D.T.A and vortex for 2-3 min
2. The homogenate was centrifuged at 15000 rpm @ 15 min at 4°C
3. Pipette out 200 µl supernatant (enzyme extract) in a test tube (duplicates)
4. Add 2ml reaction mixture to enzyme extract and make final volume to 3 ml by adding 0.8ml of ADW
5. Now the reaction was started by adding 400 µl of riboflavin to each test and placing the test tubes under two 18 W fluorescent lamps for 15 min
6. After 15 min the reaction was terminated by switching off the light, and tubes were kept in the dark
7. The observations recorded at 560 nm on a UV-vis spectrophotometer

Control: Complete reaction mixture without enzyme extract + light act as a control

Blank: complete reaction mixture without enzyme extract – light act as blank.

Calculation:

% inhibition of NBT reduction by SOD is equal to (reference control OD-treatment OD/ reference control) x 100

50% inhibition is equal to 1 unit of the enzyme.

The final formula for SOD units /g fresh weight of sample will be

$$\text{SOD Units /g FW} = \frac{C-T}{T} \text{ X } 100 \text{ X } \frac{1}{50} \text{ X } \frac{\text{System volume}}{\text{Enzyme extract}} \text{ X DF X } \frac{1}{0.1}$$

Where C = reference control OD
T = treatment OD
System volume = 3 ml + 0.4 ml = 3400 µl
Enzyme extract volume = 200 µl
DF= dilution factor = 3000/200 = 15
0.1 = starting material weight 0.1 g

3.2.9.2 Measurement of lipid peroxidation

Lipid peroxidation is the oxidative degradation of lipid-fatty acids by reactive oxygen species and hence it is considered as one of the measures of oxidative stress in the cells. The method for lipid peroxidation estimation described by Ohkawa et al. (1979) is given as follows.

Principle

Oxidative degradation of lipid-fatty acids by reactive oxygen species increases the concentration of lipid hydroperoxides and aldehydes in the cells. These lipid hydroperoxides and aldehydes react with 2-thiobarbituric acid (TBA) hence called Thiobarbituric acid reactive substances (TBARS). The TBARS content is measured in terms of malondialdehyde (MDA) which results from the decomposition of the unstable peroxides of polyunsaturated fatty acids. MDA reacts with (TBA) resulting in the formation of a red-colored complex with absorbance maxima at 532 nm (www.isca.in).

Solutions

- 20 % Trichloroacetic acid (TCA)
- Thiobarbituric acid reagent: 1% TBA in 20% TCA

Procedure

1. To 0.1 grams of powder, the leaf sample adds 4.0 ml of 20% TCA containing 1% TBA in a test tube prepared in duplicates/replicates.
2. Heat the mixture for 30 min at 95° C in a water bath and immediately cool in an ice bath to stop the reaction.
3. Centrifuge the cool samples at 10 000 g for 15 min.
4. Record the absorbance of the clear supernatant at 532 nm

Blank: A sample blank containing the 20% TCA containing 1% TBA without enzyme extracts.

Calculation: The MDA content can be calculated either from a standard curve of MDA (0- 20µM range) or based on the extinction coefficient of MDA 1.56 x 10^5 M^{-1} cm-1 or 156000 $M^{-1}cm^{-1}$. For MDA standard, replace the sample with the dilutions of MDA. The MDA content is represented as nmol per g dry or fresh weight.

Formula:

As per Beer,s Lambert Law

A = ε l c i.e. c = A/ ε l

Where A = Absorbance of solution at a particular wavelength;
ε = Molar Absorptivity or molar extinction coefficient of MDA = 156000 $M^{-1}cm^{-1}$;
l = Length of Solution Cell = here 1 cm;
c = Concentration of Solution (mmol/L).

To covert c to nmol/L/g; multiply by 10^6 and divide by 0.1 (since starting material is 0.1 g leaf powder). Therefore formula becomes

$$C = \frac{A \times 10^6}{\varepsilon \times l \times 0.1} \quad \text{nmole/L/g}$$

The same calculation can also be done using the web browser https://www.instanano.com/2017/01/Concentration-Calculation-UV-Vis-Absorbance.html

3.2.9.3 Measurement of peroxidase (POx) activity (E.C.1.11.1.7)

Peroxidase (POx) activity was measured spectrophotometrically as described by Hammerschmidt et al. (1982) with pyrogallol as a hydrogen donor.

Principle

Peroxidase also referred to as non-specific peroxidase catalyzes the reduction of hydrogen peroxide with concurrent oxidation of pyrogallol to a colored purpurgallin. The increase in absorbance is recorded at 420 nm.

$$H_2O_2 + \text{Pyrogallol (donor)} \xrightarrow{\text{peroxidase}} 2H_2O + \text{purpurgallin(oxidized donor)}$$

Solutions

0.05M Pyrogallol

0.1 M Phosphate buffer (pH7)

1% H_2O_2

Procedure

1. To 0.1 gram of powder, the leaf sample adds 5 ml ice-cold 0.1 M Phosphate buffer (pH7) in a test tube prepared in duplicates/replicates.
2. Homogenate was vortex for 2-3 min for easy dispersion of leaf sample in the buffer
3. The Homogenate was centrifuged @ 16000 rpm at 4°C for 15 min and the supernatant was used as a crude enzyme source.
4. Take 50 µl of supernatant in a test tube (replicates) and add 1.5 ml of 0.05M Pyrogallol
5. Just before taking the observation at the spectrophotometer add 0.5 ml of 1% H_2O_2
6. The observation at 420 nm was recorded at 30 s intervals for 3 min

Blank: A sample blank contains the 0.1 M Phosphate buffer, 0.05M Pyrogallol, and 1 % H_2O_2 without enzyme extracts.

Calculations

Peroxidase activity (U/L)

$$= \frac{\Delta\text{Abs} \times 100 \times \text{System volume} \times 1}{\Delta t \times € \times L\ \text{Enzyme extract} \times 0.1}$$

Where

ΔAbs = change in absorbance = Absorbance at 3 min-Absorbance at 0 min

Δt = change in time (in min, here 3 min)

L = path length or cuvette diameter (=1 cm)

€ = molar extinction coefficient of substrate in units of $M^{-1}cm^{-1}$ (here for pyrogallol its 12 $M^{-1}cm^{-1}$

Total assay volume (= 1.5 ml + 50 ul + 0.5 ml = 2050 ul)

Enzyme extract volume (= 50 ul)

3.2.9.4 Estimation of Phenylalanine ammonia-lyases (PAL) (E.C.4.1.3.5)

PAL activity was measured in terms of amounts of t-cinnamic acid (t-CA) formed according to the method of Brueske (1980).

Principle

Phenylalanine ammonia-lyase catalyzes the conversion of phenylalanine to trans-cinnamic acid and ammonia, as a step in the phenylpropanoid pathway of plants and is therefore involved in the biosynthesis of the polyphenol compounds such as flavonoids, phenylpropanoids, and lignin in plants.

$$\text{L-Phenylalanine} \longleftrightarrow \text{trans-cinnamic acid} + NH_3$$

Solutions

0.1M Phosphate buffer containing 1.4 mM mercaptoethanol

0.1 mM L-Phenylalanine (pH8.7)

1M TCA

Procedure

1. 1 gram of leaf sample was homogenized in 2ml of ice-cold 0.1 M Phosphate buffer having 1.4mM mercaptoethanol and vortex.
2. Centrifuge at 16000 rpm at 4^{o}C for 15min
3. Pipet out 200 µl of supernatant (enzyme extract) into a test tube (replicates)
4. Add 500 µl of 0.1 M phosphate buffer and 1.3 ml of ADW and mix it.

5. To initiate reaction add 500 µl of 0.1mM L-Phenylalanine in each test tube and incubate at 32^{o}C for 30min
6. The reaction was stopped by adding 500 µl of 1M TCA
7. Record the observation at 290 nm

Blank: A sample blank containing the 0.1 M Phosphate buffer, 0.1 mM L-Phenylalanine, and 1 M TCA without enzyme extracts.

Calculations

trans-cinnamic acid content can be calculated either from a standard curve of trans-cinnamic acid or based on the extinction coefficient of trans-cinnamic acid. The trans-cinnamic acid content is represented as mmole/L/g fresh weight.

Formula:

As per Beer's Lambert law

$$\mathbf{A = \varepsilon\, lc \text{ i.e. } c = A/ \varepsilon\, l}$$

Where A = Absorbance of solution at a particular wavelength

ε = Molar Absorptive or molar extinction coefficient of Trans cinnamic acid = 9630 $M^{-1}cm^{-1}$;

l = Length of Solution Cell = here 1 cm;

c = Concentration of Solution (mmol/L).

To covert c to mmole/L/g; divide by 0.1 (since starting material is 0.1 g leaf powder). Therefore formula becomes

$$\mathbf{C = \frac{A}{\varepsilon \times l \times 0.1} \quad \text{mmole/L/g}}$$

The same calculation can also be done using the web browser

https://www.instanano.com/2017/01/Concentration-Calculation-UV-Vis-Absorbance.html

3.2.9.5 Polyphenol oxidase (PPO) (E.C.1.14.18.1)

PPO activity was evaluated as described by Gauillard et al. (1993) with catechol as a substrate for PPO.

Principle

Enzymatic oxidation of catechol by PPO/O_2 transformed the substrate into a yellow product with a maximum absorbance at 495 nm. These enzymes are released by broken cells and they catalyze the reaction between colorless molecules called polyphenols and molecular oxygen this reaction creates colored compounds and these new compounds can spontaneously cross-react with one another to form black-brown complexes called melanins.

$$\text{Catechol + oxygen} \xrightarrow{\text{polyphenioxidase}} \text{Benzoquinone + water} \longrightarrow \text{melanin}$$

Solutions

0.1M Phosphate buffer containing 0.01M catechol (reaction mixture) (pH6.5)

Procedure

1. 0.1 gram of leaf sample was homogenized in 5ml of 0.1M Phosphate buffer
2. Homogenate was centrifuged at 16000 rpm for 30 min @ $4^{o}C$
3. The reaction mixture contained 0.01 M catechol (0.4 ml) in 0.1 M sodium phosphate buffer (3.0 ml; pH 6.5).
4. At the time of taking observation 400 µl of the enzyme, the extract was added to the reaction mixture and reading was taken.
5. The observation at 495 nm was recorded at 30-sec interval up to 3 min

Blank: A sample blank containing the 0.1M Phosphate buffer containing 0.01M catechol (reaction mixture) without enzyme extracts.

Calculation

<u>Polyphenol oxidase activity (U/L) = change in OD per minute per gram Fresh weight</u>

$$= \frac{\Delta\text{Abs } \mathbf{x\ 100\ x\ \ System\ volume\ x\ 1}}{\Delta \text{ t x € x L Enzyme extract X 0.1}}$$

Where

ΔAbs = change in absorbance = Absorbance at 3 min-Absorbance at 0 min
Δt = change in time (in min, here 3 min)
L = path length or cuvette diameter (=1 cm)
€ = molar extinction coefficient of substrate in units of $M^{-1}cm^{-1}$ (here for catechol its 3450 $M^{-1}cm^{-1}$
Total assay volume (0.4 ml + 3.0 ml + 400 µl = 3800 ul)
Enzyme extract volume (= 400 ul)

3.2.9.6 Total phenolic content (TPC)

TPC was determined by Zheng and Shetty (2000).

Solutions

95 % ethanol

1N Folin-ciocalteau reagent

5% Na_2Co_3

Procedure

1. 0.1 gram of leaf sample was placed in 5 ml of 95% ethanol and kept at 0^oC for 48 hrs for maximum extraction
2. Each sample was then homogenized and centrifuged at 13000 rpm for 10 min
3. Pipet out 1 ml of supernatant and mix it with 1 ml of 95 % ethanol and 5 ml ADW
4. To this added 0.5 ml of 1 N Folin-ciocalteau reagent was added
5. After 5 min added 1 ml of 5% Na_2Co_3 and the reaction mixture was allowed to stand for 60 min and the absorbance at 725 nm was recorded

The calibration curve was prepared for using various concentrations of gallic acid (GA) in 95 % ethanol. The absorbance value was converted to µg or mM gallic acid equivalent (GAE) g/FW.

Blank: A sample blank contained 95 % ethanol, 1N Folin-ciocalteau reagent, and 5% Na_2Co_3 without enzyme extracts.

3.2.10 Molecular characterization of potential fluorescent *Pseudomonas* Isolates

3.2.10.1 Procedure for genomic DNA isolation of fluorescent *Pseudomonas*

DNA extraction of potential fluorescent *Pseudomonas* isolates was done as per manufacturer's instructions using HiPurA^{TM} genomic DNA purification kit (Cat. no. K3100-02)

Contents of kit:

1. Binding buffer (B2 or B3)
2. Wash buffer (W1)
3. Elution buffer (E1)
4. PCR spin columns and collection tubes
5. Elution tubes

Buffer preparation before DNA isolations

Binding buffer (B2): Added 48ml of 100% isopropanol

Binding buffer (B3): Added 11ml of 100% isopropanol

Washing buffer (W1): Added 320 ml of 96-100% ethanol

Procedure:

1. Added 2ml of each actively growing fresh fluorescent *Pseudomonas* isolates in 2ml Eppendorf tube, and centrifuged at 3000 rpm for 3 minutes, decanted the supernatant (added more culture if formed pellet is small), and repeated the processes.
2. Resuspended the pellet thoroughly in 200µl of lysis solution (C1), vortexed thoroughly (about 15 seconds), and incubated at 55°C for 10 minutes.
3. Added 200µl of ethanol (96-100%) to the lysate and mixed thoroughly by vortexing for a few seconds.
4. Loaded lysate into HiElute miniprep spin column (capped) provided.
5. Centrifuged at 10000 rpm for 1 minute at room temperature
6. Discarded the flow-through liquid and place the spin column in the same 2ml collection tube.
7. Added 500µl of prewash solution to the column centrifuge at 10000 rpm for 1 minute at room temperature
8. Discarded the flow thorough liquid and re-used the same collection tube with the column.
9. Added 500µl of dilute wash solution (WS) to the column and centrifuged for 3 minutes at 13000 rpm at room temperature.
10. Discarded the flow-through and spin again at the same speed for the additional 1 minute to dry the column.
11. The column must be free of ethanol before eluting the DNA
12. Transferred the HiElute miniprep spin column (capped) to the fresh uncapped collection tube
13. Pipetted 200µl of elution buffer (ET) directly into the column without spilling to the sides. Incubated for 1minute at room temperature.
14. Centrifuged at 10000 rpm for 1 minute to elute the DNA.
15. Transferred the elute to a fresh capped 2 ml collection tube for further analysis.

Dilution of DNA

Dilution of the above-eluted DNA was done using Tris-EDTA buffer to make the concentration of DNA as 40-60ng and subsequently used for PCR amplification.

3.2.10.2 Procedure for making Whole-cell template (WCT) for PCR amplification for antibiotic-related genes

Procedure

Young viable cultures of potential *Pseudomonas* isolates were inoculated into 50 ml of King's B broth and incubated for 48hrs at 27°C. Then took 5µl of bacterial colonies from the incubated cultures and transferred into 95µl of lysis buffer.

Lysis buffer

Component	Stock	Working	100 ml
KCl	1M	50mM	5ml
Tris HCl (pH-8)	1M	10mM	1ml
Tween-20	-	0.1%	100 µl

*made up the final volume to 100 ml by adding ADW

The resulting suspension was centrifuged @ 12000 rpm for 1 minute. Incubated for 10 minutes at 99°C in thermal cyclers. Heat lysed bacterial suspension was frozen at -20°C for 30 minutes. After thawing 4µl volume of supernatant carefully taken for PCR analysis.

3.2.10.3 Characterization of fluorescent *Pseudomonas* using genus and species-specific loci.

To confirm the strains as fluorescent Pseudomonas, 16S-23S rRNA intervening sequence-specific primers 27F+1492R and 16S were used to get an amplicon size of 1500bp

3.2.10.3.1 ERIC-PCR-based genotypic analysis

ERIC primers were used to detect a difference in the number of repetitive sequence in the bacterial genome. This is a PCR based marker and was carried out using primer ERIC-F and ERIC-R as described by Brujin in 1992. Based on data derived from the ERIC PCR profile, a matrix with binary code comprising 1 and 0 was made for the presence or absence of band respectively. SIMQUAL program was used to compute the symmetric matrix in the form of average taxonomic distances. Sequential, agglomerative, hierarchical, and nested (SHAN) clustering was used for the cluster analysis using NTSYS software, and a dendrogram was generated.

Table: 3.1 Sequence of primer used for characterization of fluorescent *Pseudomonas* spp. using genus, species-specific, and antibiotic genes.

Primer	Gene/Reference	Sequence	Tm(°C)
27F		5’- AGAGTTTGATCMTGGCTCAG-3’	56
1492R		5’-CTACGRRTACCTTGTTACGAC-3’	56
ITS2R		5’-AAGGAGGTGATCCAGCCGCA-3’	53.2
ITS1F		5’-AGAGTTTGATCCTGGCTCAG-3’	46.4
ERIC-F	de Bruijn (1992)	5’-AAGTAAGTGACTGGGGTGAGCG-3’	62
ERIC-R		5’-TATAAGCTCCTGGGGATTCAC-3’	62
Primer		**Sequence**	**Tm(°C)**
PrnA R	Pyrrolnitrin	5’-TGCCGGTTCGCGAGCCAGA-3’	62
PrnA F	De souza and Raaijmakers (2003)	5’-GTGTTCTTCGACCTTCCTCGG-3’	62
Phl2aF	2,4Diacetyl phloroglucinol	5’-GAGGACGTCGAAGACCACCA-3’	61.4
Phl2bR	Raaijmakers et al. (1997)	5’-ACCGCAGCATCGTGTATGAG-3’	59.4

Table: 3.2 Reaction mixture for performing PCR using different primer

The reaction mixture for performing PCR using Phl 2a-2b primer (WCT)

Component	Stock concentration	Volume per reaction (20µl)
WCT	-	4 µl
ADW	-	9.5 µl
10 X Buffer	10X	2 µl
dNTP	1mM	2µl
Primer (F+R)	10 µM	2 µl(1+1)
Taq Polymerase	1U/ µl	0.5 µl

Reaction mixtures for performing PCR using 27+149, 16S, PrnA, ERIC primers

Component	Stock concentration	Volume per reaction (10µl)
DNA	-	1.5µl
PCR Water	-	5.25 µl
10 X Buffer	10X	1µl
dNTP	1mM	1µl
Primer (F+R)	10 µM	1µl (0.5+0.5)
Taq Polymerase	1U/ µl	0.25 µl

Table: 3.3 Amplification thermal profiles for primers

ERIC (Enterobacterial Repeat Intergeneric Concerns)

Steps	Activity	Temperature (^oC)	Time (min)	Repeats
1	Initial denaturation	94	3	1
2	Final denaturation	94	1	
3	Annealing	53	1	45
4	Extension	72	1	
5	Final extension	72	8	
6	Storage	4		

PrnA (Pyrrolnitrin)

Steps	Activity	Temperature (^oC)	Time (min)	Repeats
1	Initial denaturation	95	3	1
2	Final denaturation	95	1	
3	Annealing	62	1	35
4	Extension	72	1	
5	Final extension	72	5	1
6	Storage	4		

PHL 2a-2b (2, 4 diacetylphloroglucinol)

Steps	Activity	Temperature (^oC)	Time (min)	Repeats
1	Initial denaturation	95	3	1
2	Final denaturation	95	1	
3	Annealing	60	1	34
4	Extension	72	1	
5	Final extension	72	5	1
6	Storage	4		

27+149, 16S-23S rRNA intervening sequence-specific internal Transcribed (ITS)

Steps	Activity	Temperature (^oC)	Time (min)	Repeats
1	Initial denaturation	94	5	1
2	Final denaturation	92	4	
3	Annealing	40	1	1
4	Extension	72	2	

5	Final extension	72	10	1
6	Storage	4		

3.2.11 Polyacrylamide gel electrophoresis (PAGE) for visualization of PCR product (CBS scientific dual vertical unit was used for the preparation of PAGE)

1. Cleaned the glass plates thoroughly with mild detergent and wiped it using a paper towel. After complete drying of plates applied gasket around the sides of rectangular backplates starting from one end. Kept the flat side of the gasket on the bottom side of the plate.
2. Aligned the notches of the gasket around the rounded corners of the glass plate. Once the gasket was pushed over the bottom edge and corner, worked it down the remaining side.
3. Placed the gasket plate on a flat surface with the tubing side up and placed the spacer on two sides of the plate (rounded corner of the spacer should face outside the bottom of the plate).
4. Placed the notched plate on top of the bottom assembly and placed two clamps on the bottom of the plate assembly.
5. Lifted the assembly to stand it on the base of the clamp. Pushed the plates down to level the assembly. Clamp the sides of plates.
6. PAGE solution (70 ml of 5% PAGE, 800µl of freshly prepared 10% ammonium persulphate (APS) solution, and 80µl TEMED (N-N-N-N tetramethyl ethylene diamine) were introduced gently to the gel plate sandwich using a notched beaker or syringe. Inserted the comb and allowed the gel to solidify.
7. After 30 minutes disassembled the clamps and gasket
8. Positioned of plate set in electrophoresis unit with notched plate facing the upper buffer reservoir and clamp-on sides. If running one side of the dual plate assembly, use an unnotched backplate to seal the open side of the upper reservoir.
9. Poured freshly prepared buffer in upper and lower chambers. Remove the combs and load the gel samples mixed with dye.

10. Aligned the power leads, attached safety cover, and connected to the power supply. Run at 150-200 volts for 20-30 minutes.
11. After the run completes, switched off the power and disassembled the glass plates.
12. Removed the side clamps and pulled the notched plate outside taking into care that gel sticks to the glass plate.
13. Detached the gel carefully without break for staining with 1XTBE buffer containing 4µl/100 ml ethidium bromide (10mg/ml). Stained for 5-10 minutes; rinse the gel with distilled water. The gels were scanned and visualized the band in gel documentation system or *BIO-RAD* gel doc XR+

Note: Complete care was taken while using TEMED and staining with ethidium bromide solution as they are carcinogenic and mutagenic agents, respectively.

3.2.12 Sequencing of PCR product

PCR products were purified by using MoBIO to make the kit as per the manufacturer's instructions to remove the PCR impurities. Purified PCR products were sent to Eurofins laboratory Bangalore for Sanger's sequencing. FASTA sequences so obtained were subjected to BLASTN analysis using NCBI public domain to identify species of fluorescent *Pseudomonas*.

3.2.13 Protocol used for purification of amplicons:

1. The PCR product was transferred into a 2ml Eppendorf tube and to it, 5 volume of spin bind was added after shaking the spin bind before use.
2. The contents were mixed well by pipetting.
3. PCR/Spin bind mixture was later transferred to a spin filter unit.
4. The spin filter units were centrifuged at 13000 rpm for about 1minute.
5. The spin filter basket was removed and flow-through was discarded by decanting and the basket was placed back into the same tube.
6. Later 300µl spin clear buffer was added to the spin filter and centrifuged at 13000rpm for about 1 minute
7. The spin filter basket was removed and flow-through was discarded by decanting and the basket was placed back into the same tube.
8. The spin filter units were again centrifuged at 13000 rpm for 1 minute.
9. The spin filters were then transferred into clean 2ml collection tubes.

10. 50µl of elution buffer solution was added onto the center of the white spin filter membrane and then centrifuged the tubes 13000 rpm for 1minute.
11. The spin filter basket was discarded from the spin filter unit and purified DNA was collected in the 2 ml collection tube and the DNA was stored at -20°C. Later purified DNA was sent for sequencing.

3.2.14 Data analysis and Statistical analysis

1. Based on data derived from the carbon source utilization profile, a matrix with binary code composing positive (+) and negative (-) values was made. SIMQUAL program was used to compute the symmetric matrix in the form of average taxonomic distances. Sequential, agglomerative, hierarchical, and nested (SHAN) clustering was used for the cluster analysis using NTSYS software.
2. All field and pot experiments were carried out in rainout shelter and college farm fields arranged in completely randomized design and randomized block design respectively with three replications in each treatment.
3. The data (biochemical and enzymatic) were subjected to statistical analysis using WASP (Web Agri Stat Package) (http://icargoa.resin/wasp/idex.php). The critical difference at 0.01and 0.05% level of significance was calculated for the observed values along with average and standard error.

Table: 3.4 Composition of different media used in the present investigation

King's B Base medium	
Dipotassium hydrogen orthophosphate	1.5g
Agar	20g
Magnesium sulfate	1.5g
Protease Peptone	20g
Distilled water	1000ml
Glycerol	15ml
Gelatin broth (pH 6.8)	
Peptone	5g
Yeast extract	3g
Distilled water	1000l
Gelatin	1200g
Potato Dextrose Agar medium	
Potato	250g
Dextrose	20g
Agar	20g
Distilled water	1000ml
Nitrate Broth (Himedia)	

Beef extract	3g/l
Peptone	5g/l
Potassium nitrate	1g/l
Sulphanilic acid (HIMEDIA)	1g/l
Zinc (HIMEDIA)	1g
Final pH at 37°C	7.0±0.2
Minimal medium, DF salt (Dworkin and Foster,1958)	
Na_2HPO_4	3g/l
KH_2PO_4	5g/l
Gluconic acid	2g/l
Glucose	1g/l
$MgSO_4.7H_2O$	0.2g
Citric acid	2g
$FeSO_4.7H_2O$	7.0±0.2
$(NH_4)_2PO_4$	0.001g
$MnSO_4.H_2O$	11.19µg
H_3BO_3	1mg
$CuSO_4.5H_2O$	78.22µg
$ZnSO_4.7H_2O$	124.6 µg
MoO_3	10µg
Distilled water	1000ml
Pikovskaya's agar medium	
Glucose	10g
$Ca_3(PO_4)$	5g
NaCl	0.2g
KCl	0.5g
$MgSO_4.7H_2O$	0.1g
$NH_4(SO_4)$	0.5g
$FeSO_4.7H_2O$	2mg
Yeast extract	0.5g
$MnSO_4.7H_2O$	2mg
Agar	15g
Distilled water	1000ml

3.2.15 Solution used for different test

3.2.15.1 Chrome azurol sulfonate (CAS) solution

1. **1M HCl (stock):** 0.36ml of conc. HCl diluted in 10ml distilled water
2. **1M $FeCl_3$ $6H_2O$ (stock):** 2.703g $FeCl3.6H_2O$
3. **Iron (Fe)(III) solution**

Chemical	Stock solution	Working concentration	Volume required per 1000ml
$FeCl_3.6H_2O$	1M	1Mm	10µl
HCl	1M	1mM	100 µl

Both components were mixed and made up the volume to 10ml with ADW

Chrome azurol sulphonate solution

50ml of autoclave distilled water was poured in a beaker. To the beaker 60.5mg of CAS and 10ml of iron (III) solution was added and mixed well by stirring. HDTMA solution (in 40ml autoclaved water 72.9mg was dissolved). This resulted in the formation of a dark blue liquid.

Salkowski's reagent

S.No	Component	Amount (g/l)
1	$FeCl_3 6H_2O$	7.5ml of 0.5M
2	Conc.H_2SO_4	150ml
3	ADW	250ml

3.2.15.2 Reagent required for inorganic phosphate quantitative estimation

1. **Chloromolybdic acid:** In 150ml ADW 7.5g of ammonium molybdate was dissolved. Then it was mixed into 162ml of conc.HCl. The final volume was adjusted to 1000ml with autoclaved distilled water and it was stored in an amber-colored bottle at 4°C.
2. **Chlorostannous acid:** In 100ml of concentrated HCl dissolved 25g of stannous chloride and adjusted the final volume to 1000ml by adding ADW and store at 4°C in an amber-colored bottle.

3.2.15.3 Buffers, Solutions, and Reagents

Composition of buffers used in antioxidant enzyme extraction assay

Component	Stock	Grams	ADW (ml)
$NaH_2PO_4 2H_2O$	1M	7.8g	50
Na_2HPO_4	1M	7.1g	50

NOTE: Above two stocks were prepared separately in 50 ml tarson tubes and acidic pH was adjusted by using phosphoric acid.

0.1M Phosphate buffer

Component	Stock	Volume from stock (ml)	ADW (100ml)
(X)$NaH_2PO_4 2H_2O$	1 M	4.24	100
(Y)Na_2HPO_4	1M	5.78	

NOTE: Both component X and Y were mixed in a beaker and the final volume was adjusted by adding ADW up to 100 ml and prepared in duplicates with pH 7.0, pH 6.5 respectively.

5 % Sodium carbonate: Dissolved 10 grams of Na_2CO_3 in 180 ml of ADW to get a clear solution and final volume adjusted to 200ml by adding ADW.

20 % Trichloroacetic acid (TCA) containing 1% Thiobarbituric acid (TBA)

Component	Grams	ADW (ml)
20 % TCA	20	80
1 % TBA	1	10

NOTE: Mixed by stirring above both the components in the beaker and keep the beaker in a water bath @ 54°C for 45 min and make up the final volume to 100 ml by adding ADW.

3M Sodium carbonate: Dissolved 15.9 grams of Na_2CO_3 in 50 ml of ADW

0.1M Phosphate buffer containing 0.5mM E.D.T.A.

Component	Stock	Working	Volume for 100 ml
Phosphate buffer	1 M	0.1 M	10 ml
E.D.T.A.	0.5 M	0.5mM	100 µl

NOTE: Mixed above both components in a beaker and added 80 ml of ADW and adjusted pH 7.5 and then made up the final volume to 100ml by adding ADW.

3.2.15.4 Solutions prepared Freshly: Following enzymes/stock buffers were prepared freshly at the time of the experiment.

Ice cold 0.1M Phosphate buffer containing 1.4mM β-Mercaptoethanol

Component	Stock	Working	50 ml
Phosphate buffer	1 M	0.1 M	50ml
β-Mercaptoethanol	-	1.4mM	5 µl

NOTE: Take 50 ml of 0.1 M phosphate buffer in 50 ml tarson tube (pH: 7) and add 5 µl of β-Mercaptoethanol carefully and mix it and store it in 4°C to make it ice cold.

0.1mM L-Phenylalanine: Dissolve 0.825 grams of L-Phenylalanine in 40 ml of ADW and adjust pH-8.7 with 0.1N KOH and makeup to 50ml by ADW.

0.01M Catechol: Dissolve 0.11 grams of Catechol in 100 ml of 0.1 M phosphate buffer (pH6.5)

0.05M Pyrogallol: Dissolve 0.630 grams of pyrogallol in 100 ml of 0.1 M phosphate buffer (pH7)

200mM Methionine: Dissolve 2.784 grams of methionine in 20 ml of 1N HCL and store it at -4°C

3.2.15.5 Solution for genomic DNA extraction

1. **1M Tris-HCl solution (pH 8.0)**

   121.1g Tris-HCl was dissolved in 800ml distilled water. Volume was made up to 1000ml with distilled water and was sterilized by autoclaving after adjusting the pH to 8 with HCl.

2. **0.5M EDTA solution (pH8.0)**

   186.1g EDTA was dissolved in 800ml distilled water. The solution was stirred vigorously on a magnetic stirrer and the pH was adjusted to 8 by adding NaOH pellets. The final volume was made up to 1000ml with distilled water followed by sterilizing in autoclaving.

3. **Chloroform**
4. **Ethanol (100% and 70%)**
5. **isoamyl alcohol**
6. **Isopropanol**
7. **TE buffer (pH 8.0)**

   10ml 1M Tris HCl and 2ml of 0.5M EDTA were mixed and the final volume was made up to 1000ml with ADW

8. **TES buffer containing the following content**

   10mM Tris HCl
   10mM EDTA
   2%SDS

3.2.15.6 Stock and working solution for PAGE

1X TBE

S.no	Component	Conc. Required	Quantity
1	Tris Base	89mM	10.8g
2	Boric acid	89mM	5.5g
3	0.5M EDTA (pH 8.0)	2mM	4ml

Mix above all components and adjusted the volume to 100ml with distilled water

1X TBE: Dilute 10ml of 10X TBE to 100ml with distilled water.

Polyacrylamide Stock solution (40%): 2g of Bis-acrylamide and 38g of acrylamide was dissolved in 100 ml of autoclaved distilled water then it was filtered through Whatman filter paper no.1. It was stored in a brown bottle for further use.

5% Polycrylamide gel preparation

S.no	Component	Final volume
1	40% polyacrilamide solution	10.8g
2	10% APS	700µl
3	TEMED	70µl

The staining solution was prepared by mixing the following components

S.no	Stock conc.	Final volume
1	Ethidium bromide	4µl
2	1X buffer	100ml

10% APS Solution was prepared by mixing the following components

S.no	Component	Final volume
1	Ammonium persulfate	1.0 g
2	Distilled water	10ml

CHAPTER-IV

RESULTS AND DISCUSSION

Fluorescent *Pseudomonas* are aerobic, gram-negative rod-shaped bacteria inhabiting soil, emerged as a promising group of rhizobacteria which posses many characters that makes them well suited as a plant growth promoter and biocontrol agent (Weller 1988; Lemanceau 1992; Weller et al. 2002; Weller 2007; Singh et al. 2012; Singh et al. 2013). A large number of secondary metabolites (Leisinger and Margraff 1979), growth hormones (Brown 1974), antibiotics (Weller et al. 2002), and chelating compounds such as siderophores (Leong 1986) are known to be released by fluorescent *Pseudomonas*. *P. putida* shows a diverse spectrum of metabolic versatility and niche-specific adaptations (Rojo 2010; Wu et al. 2011). They maintain soil health by employing a wide variety of mechanisms, including N2 fixation, enhanced solubilization of phosphate, and phytohormone production (auxin and cytokinin). PGPR completely colonize plant roots and stimulate plant growth and reduce the incidence of plant disease. In the present study entitled **"Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters and induction of antioxidant enzymes in soybean, chickpea and wheat"** we isolated and characterized fluorescent *Pseudomonas* derived from soil and *Brassica* specific root rhizosphere. Potential isolates or PGPR strains are required for fundamental research. Through confrontation assay potential fluorescent *Pseudomonas* isolates were identified against *Macrophomina phaseolina.* The impact of the interaction of microbial populations in the root-soil interface on pre- penetration activity, infection, and resulting disease is a critical area that has received relatively little attention. In the present study, we perform bipartite and tripartite interactions assay involving fluorescent *Pseudomonas* at the root-soil interface in semi-artificial experimental set-ups. Soil bacteria in particular can grow rapidly and utilize a very wide range of different substances as nutrient sources. Direct promotion of plant growth by plant growth-promoting rhizobacteria by facilitating the acquisition of nutrient resources from the environment including nitrogen fixation, siderophore production, phosphate solubilization or specifically modulating plant growth by altering plant hormones like cytokinin, ethylene, and auxin (Glick 2012). Plant growth-promoting response of potential isolates was evaluated on soybean, chickpea, and wheat. Purified fluorescent *Pseudomonas* cultures from different locations helped us to drive the component of molecular (16S–23S rRNA intervening DNA sequences) characterization.

4.1 Collection and isolation of fluorescent *Pseudomonas* from soil and *Brassica* specific rhizosphere.

Rhizospheric soil of different crop plants derived from different locations of Bastar and Bilaspur districts of Chhattisgarh for isolation of fluorescent *Pseudomonas* (Plate: 4.1). Fluorescent *Pseudomonas* isolated from the *B. napus* rhizosphere was shown to possess strong antifungal and plant growth-promoting properties (Kalbe et al. 1996; Larcher et al. 2008; Njoroge et al. 2008) which prompted us to derive soil from *Brassica* specific root rhizosphere. Soil samples were passed through a sieve to remove debris. Isolation of bacteria was done by standard serial dilution technique on King's B agar media and plates were incubated at 28^{o}C for 48 hr (Plate 4.2). Single bacterial colonies of fluorescent *Pseudomonas* were detected under the UV light (366nm) and further purified by streaking on to the Kings'B media plates. Minute fluorescent bacterial colonies generating from few bacterial cells were derived and were subcultured and maintained on King's B agar slants for future use. Ten isolates emitting very strong fluorescence on Kings B media out of 88 were selected (Table: 4.1).

4.2 Metabolic profiling of selected fluorescent *Pseudomonas* isolates

Plant roots liberate organic compounds to the soil, a process called rhizodeposition (Whipps, and Lynch 1985). Microorganisms established in the soil ecosystem due to high adaptability in a wide variety of environments, PGPR metabolizes a variety of natural and xenobiotic chemicals. As early as 1965, Rovira suggested that microbes were stimulated in response to root exudates. Roots exude a diverse repertoire of metabolites, including up to 10–50% of the plants' fixed carbon (Jones and Nguyen, 2009, Kuijken et al., 2015, Massalha et al., 2017). Plant secondary metabolites are exuded in sufficient amounts to establish a rhizosphere biased to a rhizobacterial strain. The carbon utilization profile of fluorescent *Pseudomonas* differs accordingly (tomato and flax) from which they were isolated (Lemanceau et al., 1995). We reasoned that the fluorescent *Pseudomonas*

Plate: 4.1 Locations for *Pseudomonas* sample collection

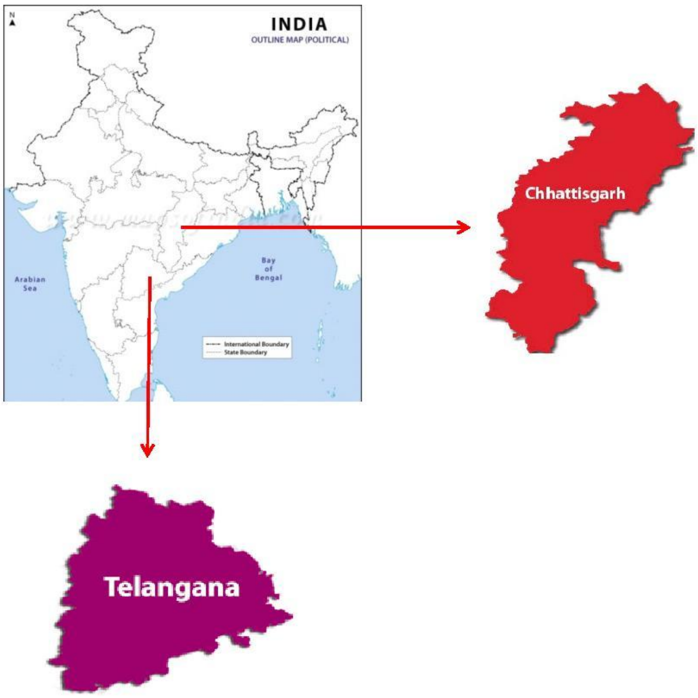

Table: 4.1 Details of fluorescent *Pseudomonas* isolates from different soil sample collection sites

S.No	Isolate	Origin/location
1	BS-1	*Brassica juncea*/ Zhahirabad
2	BS-2	*Brassica juncea* / Zhahirabad
3	BS-3	*Brassica juncea*/ Zhahirabad
4	BS-4	*Brassica juncea*/ Zhahirabad
5	9704	Jagdalpur
6	9829	Jagdalpur
7	9809	Jagdalpur
8	BSP-14	Bilaspur
9	BSP-19	Bilaspur
10	BSP-23	Bilaspur

Plate: 4.1 Locations for *Pseudomonas* sample collection

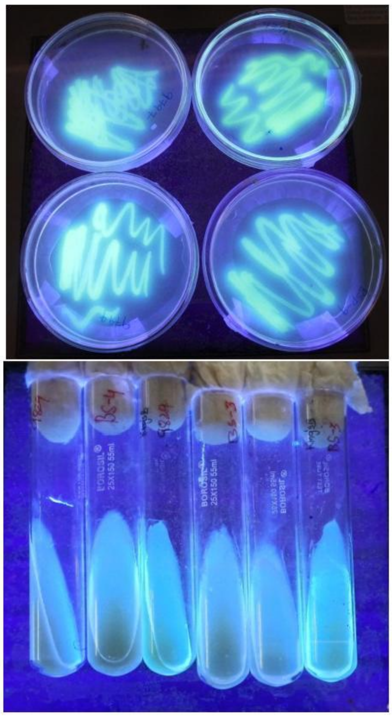

Plate: 4.2 Isolation of fluorescent *Pseudomonas* from soil and *Brassica* specific rhizosphere.

Plate: 4.2 Isolation of fluorescent *Pseudomonas* from soil and *Brassica* specific rhizosphere.

preadapted to plant roots liberate organic compounds will express a similar metabolic profile. Nevertheless, the issue prompted us to screen selected ten isolates of fluorescence *Pseudomonas* (Bs-1, Bs-2, Bs-3, Bs-4, 9704, 9829, 9809, BSP-14, BSP-19, BSP-23) for their metabolic profiles (carbon utilization on HiCarbo Kit, Starch hydrolysis, Gelatin liquefaction, Nitrate reduction, Growth at 42^{o}C, Growth at 4^{o}C, Carbenicillin and Kanamycin sensitivity) (Table 4.2).

4.2.1 Starch hydrolysis

Microbes use starch as a source of carbon and energy for growth. The amylase enzyme catalyzes starch. In our study, *Pseudomonas* isolates were tested for amylase producing ability by performing the agar test to determine the absence and presence of iodine produces a dark blue coloration and a yellow zone around the colony in an otherwise blue media indicates amylolytic activity. Luxuriant growth and formation of the yellow zone around the isolates after flooding with Lugol's iodine solution were observed for eight isolates viz., Bs-1, Bs-2, Bs-3, Bs-4, 9809, 9829, 9704, and BSP-14. Two isolates(BSP-19 and BSP-23) were found not hydrolyzing starch (Plate: 4.3a). Generally, starch hydrolysis is one of the characteristic features of P. *aeruginosa*.

4.2.2 Gelatine liquefaction test

Gelatin is a protein derived from collagen, proteolytic microbes digest protein and may liquefy gelatin. The gelatification of gelatin in the index of the proteolytic activity of the microbes produces the enzyme gelatinase. Rapid gelatin hydrolysis was observed for the following eight *Pseudomonas* isolates viz., BS-1, BS-2, BS-3, BS-4, 9809, 9829, 9704, and BSP-14 (Plate: 4.3b). Rapid gelatin liquefaction is one of the differentiating features of *P. aeruginosa*. In two isolates (BSP-19 and BSP-23) semi solidified gelatin agar medium was observed after incubation at 4^{o}C in a refrigerator. *P. putida* does not hydrolyze gelatin.

4.2.3 Nitrate reduction test

The nitrate reduction test is a test to identify bacteria to the genus level. Bacteria producing nitrate reductase enzyme convert nitrate (NO3) to nitrite (NO2). In *in vitro* reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-naphthyl amine. Nitrite reacts with acid solutions and forms red azo dye (diazonium salt). Thus, red dye indicates the presence of NO-2 (nitrite). In the

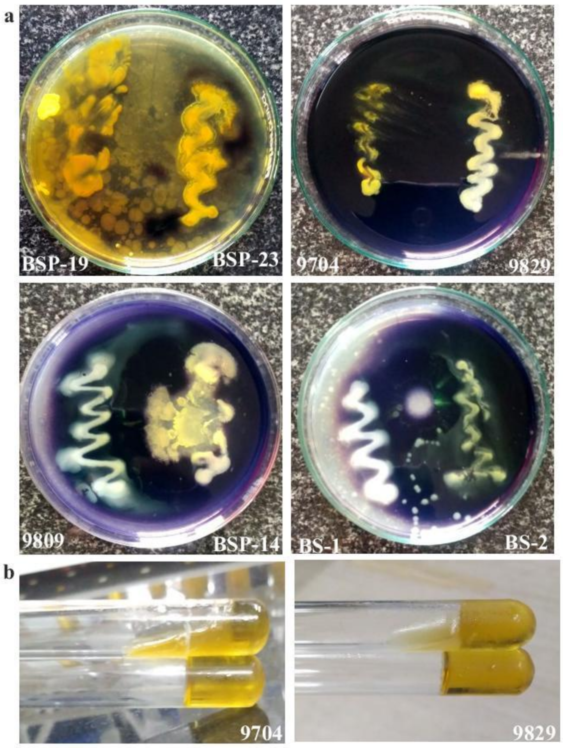

Plate: 4.3 Biochemical characterization of fluorescent *Pseudomonas* isolates
a. Starch hydrolysis test; b. Gelatin liquefaction test

present study, *Pseudomonas* isolates were tested for nitrate reduction in nitrate broth. Before the addition of the zinc powder (reducing agent) to the broth, nitrate reduction was shown by 80 % of total isolates. But after the addition of a pinch of zinc powder, no change in color was observed with 20 % of isolates. Among 10 *Pseudomonas* isolates 8 isolates viz., Bs-1, Bs-2, Bs-3, Bs-4, 9809, 9829, 9704 and BSP-14 produced red color before the addition of zinc powder which is a differentiating feature of *P. aeruginosa*. Two isolates BSP-19 and BSP-23 showed no change in the color of broth is observed even after the addition of zinc powder which is a differentiating feature of *P. fluorescens.*

4.2.4 Growth at 4^{o}C 42^{o}C

Historically growth of *Pseudomonas* isolates at 42^{o}C has been considered to be a key differential test for separating non-pigmented *P. aeruginosa* and other species of fluorescent *Pseudomonas*. In the present investigation, eight isolates of *Pseudomonas* viz., Bs-1, Bs-2, Bs-3, Bs-4, 9809, 9829, 9704, and BSP-14 were grown on Kings B media and showed positive growth when incubated at 42^{o}C which is a differentiating feature of *P. aeruginosa.* Whereas, isolates BSP-19 and BSP-23 showed positive growth on Kings B media when incubated at 4^{o}C which is a differentiating feature of *P. fluorescence*.

4.2.5 Antibiotic sensitive test

Sensitivity against two antibiotics, carbenicillin and kanamycin were tested for all Pseudomonas isolates. 80% of isolates were able to grow in kanamycin supplemented medium and 20% were able to grow in the carbenicillin supplemented medium. Out of 10, 8 isolates resistant to kanamycin were Bs-1, Bs-2, Bs-3, Bs-4, 9809, 9829, 9704, and BSP-14. The 2 isolates resistant to carbenicillin were BSP-19 and BSP-23. Generally, carbenicillin sensitive but kanamycin-resistant isolates belong to species *P. aeruginosa*, here Bs-1, Bs-2, Bs-3, Bs-4, 9809, 9829, 9704 and BSP-14 may be *P. aeruginosa*. However, carbenicillin resistant but kanamycin sensitive isolates belong to species *P. fluorescens* or *P. putida*. Isolates BSP-19 and BSP-23 may be either *P. flurescens* or *P. putida*.

Based on reports of Stanier et al. (1966) and Blazevic et al. (1973) some tests may help differentiate species of *Pseudomonas*. Blazevic et al. (1973)

suggested that kanamycin sensitivity or resistance to carbenicillin would provide a means of identifying *P. fluorescens, P. putida,* and separating them from *P. aeruginosa.* In the present investigation isolate Bs-1, Bs-2, Bs-3, Bs-4, 9809, 9829, 9704, and BSP-14 satisfies most of the differentiating characters of species *P. aeruginosa* (positive for nitrate reduction, gelatin hydrolysis, growth at 42°C and more importantly antibiotic test viz. kanamycin-resistant/ carbenicillin susceptible), and therefore is designated as *P. aeruginosa* (Table: 4.2). Similarly, *Pseudomonas* isolate BSP-19 and BSP-23 justifies most of the distinguishing characteristics of *P. fluorescens* (positive for nitrate reduction, gelatin liquefaction) therefore they are designated as *P. fluorescens*. Among 10 isolates none of the isolates showed a positive test for *Pseudomonas putida*. The species designation done using biochemical parameters may be confirmed by amplicon-based sequencing of DNA derived from the isolates using ITS based primers.

The results obtained in this investigation are following the reports of Blazevic et al. (1973) studied biochemical characterization and antibiotic sensitivity of fluorescein producing strain of *Pseudomonas* by rapid tests for nitrate reductase, gelatin test, and antibiotic sensitivity test for differentiating the strain of fluorescent strains (*P. fluorescens, P. putida, P. aeruginosa*). Lemanceau et al. (1995) had used ten substrates to differentiate *Pseudomonas* isolates of tomato and flax. Most of the species designation done in the present investigation is as per Stanier et al. (1966) keys.

4.2.6 HI-carbohydrate ™ kit test for identifying the differential utilization of the carbon sources by fluorescent *Pseudomonas.*

Fluorescent *Pseudomonads* are the most extensively studied subgroup of aerobic *Pseudomonads*. Den Dooren de Jong (1926) was the first to report that fluorescent *Pseudomonads* are nutritionally versatile, and can use a very wide range of simple organic compounds as sole sources of carbon and energy. Differential utilization of various types of carbohydrates as energy sources has been observed among microorganisms especially bacteria. It is known that bacteria do not utilize all available energy sources with the same efficiency. Some substances are more readily utilized than others and in the presence of these substances; the less easily utilizable substances are left unutilized.

Table: 4.2 Distinguishing tests for differentiating *P. aeruginosa*, *P.fluorescens,* or *P. putida*

	BS-1	BS-2	BS-3	BS-4	9704	9829	9809	BSP-14	BSP-19	BSP-23
Starch hydrolysis	Y	Y	Y	Y	Y	Y	Y	Y	No	No
Gelatin liquefaction	Y	Y	Y	Y	Y	Y	Y	Y	No	No
Nitrate reduction	Y	Y	Y	Y	Y	Y	Y	Y	No	No
Growth at 42^oC	Y	Y	Y	Y	Y	Y	Y	Y	No	No
Growth at 4^oC	No	No	No	No	No	No	No	No	Y	Y
Carbenicillin	S	S	S	S	S	S	S	S	R	R
Kanamycin	R	R	R	R	R	R	R	R	S	S

S: Susceptible; R: Resistant; Y: (YES) positive; NO: No growth

Several scientists such as Hugh & Leifson (1953), Stanier et al (1966), Bossis et al (2000) reported differential utilization of carbohydrates for taxonomic characterization of *Pseudomonads*.

Ten isolates were screened for carbohydrate utilization (Table: 4.3). Mannose (is a sugar monomer of the aldohexose series of carbohydrates and a C-2 epimer of glucose), Citrate (a tricarboxylic acid tri-anion), Malonate (a three-carbon dicarboxylic acid) was utilized by all the 10 isolates. Only one isolate BS-4 was able to utilize lactose. Lactose is a disaccharide sugar composed of galactose and glucose subunits. Inulin, a type of oligosaccharide called a fructan (a chain of fructose molecules strung together) was used by only 9829 isolates. Rhamnose (a naturally occurring deoxy sugar) was utilized by only BSP-19.

All the isolates except BSP-19 utilized Dextrose, Galactose (an aldohexose monosaccharide). Among 10 isolates, 70% gave a positive response for Melibiose (a reducing disaccharide formed by an α-1, 6 linkages between galactose and glucose) and L-arabinose (an aldopentose – a monosaccharide containing five carbon atoms, and including an aldehyde functional group) utilization. Three isolates 9829, BSP-14, and BSP-19 tested negative for melibiose and L-arabinose. Five isolates BS-1, BS-2, BS-3, 9704, and BSP-19 utilized ONPG (ortho-Nitrophenyl-β-galactoside is a colorimetric and spectrophotometric substrate for detection of β-galactosidase activity). D- arabinose (an aldopentose) as a carbon source was utilized by three isolates BS-1, BS-4, BSP-23. Esculin, a water-soluble glycoside, is hydrolyzed by certain bacteria to yield glucose and esculetin. Esculetin reacts with the ferric ions to produce a black colored complex that surrounds the colonies. Three isolates 9704, 9829, BSP-19 tested positive for esculin hydrolysis. Isolate BS-1 and 9704 utilized the maximum number of carbohydrates (10 out of 35 in the kit) followed by BS-2, BS-3, BS-4 which gave a positive response for 9 carbohydrates. However isolate BSP-14 utilized only 5 carbohydrate sources (Plate: 4.4)

All the 10 isolates gave negative response for the utilization of the following carbocyclic sugars or sugar alcohols or polyols: Dulcitol (derived through reduction of galactose), Inositol, Sorbitol (obtained by reduction of glucose), Adonitol (pentose alcohol formed by the reduction of ribose which is metabolized to teichoic acids used in the cell walls of gram-positive bacteria), Erythritol and Xylitol.

All the 10 isolates were unable to utilize α-methyl- D-glucoside (a monosaccharide derived from glucose), Melezitose (a non-reducing trisaccharide sugar), α-methyl-D-mannosidase (a methyl mannose having alpha-configuration at the anomeric center) and Sorbose (ketose monosaccharides), Arabitol (sugar alcohol), Raffinose (a trisaccharide composed of galactose, glucose & fructose) and Trehalose (a non-reducing sugar composed of two molecules of glucose), Maltose (a disaccharide composed of two glucose units joined by α bond) & Salicin (an alcoholic β-glucoside). Fructose (a simple ketonic monosaccharide), Sucrose a disaccharide composed of two monosaccharides: glucose and fructose, Sodium gluconate which is the sodium salt of gluconic acid, Glycerol (a simple polyol compound).

Among factors affecting bacterial colonization of roots, the plant is postulated to play a major role (Glandorf, 1992). Plant roots liberate organic compounds to the soil, a process called rhizodeposition (Whipps, and Lynch 1985). The exudation of these compounds is responsible for the rhizosphere effect (Lynch, and Whipps 1990). As early as 1965, Rovira suggested that root exudates play a key role in the selective stimulation of microorganisms. The effect of specific compounds on the selection of rhizospheric microflora was first demonstrated by Gunner et al. (1966), who showed that the dominant bacteria in the rhizosphere of bean plants sprayed with an organophosphate insecticide were those able to use the insecticide metabolites as a nutrient source. Since then, other studies have reported the effect of plants on soilborne microflora (Azad et al. 1985, Kremer et al., 1990, Martin, 1971, Miller et al.1989) or introduced strains of fluorescent *Pseudomonads* (Howie, and Echandie 1983., Weller, 1986). However, information is lacking about the characteristics of soilborne fluorescent *Pseudomonads* selected by the plant (Glandorf et al. 1993, Van Peer et al. 1990). The composition of root exudates is known to differ according to plant genotypes, the contribution of the ability to use specific organic compounds to rhizosphere competence may differ between rhizospheres of different species. Carbohydrate utilization profile of fluorescent *Pseudomonas* spp. differs accordingly to the rhizosphere from which they were isolated (Lemanceau et al., 1995). Very initial studies such as that of (Liu 1952) identified that *Pseudomonas aeruginosa* has a definite biochemical pattern. They can utilize glucose, galactose, fructose, mannitol, trehalose, and glycerol as the sole source of carbon for growth and to produce acid from these substances. Therefore all isolates except BSP-19 may be *P. aeruginosa*. However other tests such as gelatin hydrolysis, nitrate reduction, starch hydrolysis, the antibiotic test showed similar results. All isolate except BSP-19 and BSP-23 are carbenicillin sensitive but kanamycin-resistant which characteristic feature of *Pseudomonas aeruginosa*. The organism cannot utilize rhamnose as the sole source of carbon for growth and will not attack it until all the other available carbon sources are exhausted hence, BSP-19 isolate did not fall into any of the phenograms and revealed all together distinct identity (Plate: 4.4). Similar results of varying responses towards carbohydrates were observed by Agrawal et al (2015) among *P. putida* isolates. Many important metabolites and commercial products can be synthesized by using carbohydrates as a primary substrate (Meur et al. 2012). Changes in the composition of metabolite diversity may affect the patterns and activities of rhizobacterial populations which are dependent upon rhizospheric nutrients for growth. Broad-spectrum carbon source utilization among *Pseudomonas* isolates may help in developing and designing stimulators for a specific application.

4.2.7 Standard data for selected nutritional and general phenotypic characteristics of selected fluorescent *Pseudomonas*

Standard data for selected nutritional and general phenotypic characteristics as given in Bergey's Manual of Systematic Bacteriology (Palleroni (1984) (Lactose, d-Xylose, maltose, d-Fructose, d-Glucose, d-Galactose, Trehalose, Sucrose, l-Arabinose, d-Mannose, inulin, glycerol, salicin, m-Inositol, Sorbitol, Mannitol, Adonitol, Erythritol, Cellobiose, d-Arabinose, citrate, Malonate, Gelatin liquefaction, Denitrification, Growth at 4°C, Growth at 41°C) for selected fluorescent *Pseudomonas* species (*P. aeruginosa, P. fluorescence* bv. I, bv. II, bv. III, bv. IV, bv. V, *P. putida* biovar A, biovar B) were used (Table: 4.4) was utilized to compare the metabolic profiles resolved by 10 isolates used in the present investigation. (Palleroni, N. J. (1984). Genus I. *Pseudomonas* Migula 1894, 237. In

Table: 4.3 Differential utilization of carbon sources by isolates of *Pseudomonas* as identified by Hi-carbohydrate kit test

TEST	**BS-1**	**BS-2**	**BS-3**	**BS-4**	**9704**	**9829**	**9809**	**BSP-14**	**BSP-19**	**BSP-23**
1	-	-	-	+	-	-	-	-	-	-
2	+	+	+	+	+	+	+	-	-	+
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	-	+
6	+	+	+	+	+	+	+	+	-	+
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	-	-	+
10	-	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	-	+	-	-	+
12	+	+	+	+	+	+	+	+	+	+
13	-	-	-	-	-	+	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	+	-
26	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-
30	+	+	+	-	+	-	-	-	+	-
31	-	-	-	-	+	+	-	-	+	-
32	+	-	-	+	-	-	-	-	-	+
33	+	+	+	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+	+	+	+
35	-	-	-	-	-	-	-	-	-	-

1. Lactose, 2. Xylose, 3. Maltose, 4. Fructose, 5. Dextrose, 6. Galactose, 7. Raffinose, 8. Trehalose, 9. Melibiose, 10. Sucrose, 11. 1-arabinose, 12. Mannose, 13. Linulin, 14. Sodium gluconate, 15. Glycerol, 16. Salicine, 17. Dulcitol, 18. Inositol, 19. Sorbitol, 20. Mannitol, 21. Adonitol, 22. Arabitol, 23. Erythritol, 24. A-methyl D-glucoside, 25. Rhamnose, 26. Cellobiose, 27. Melizitose, 28. A-methyl D-mannoside, 29. Xylitol, 30. ONPG, 31. Esculin hydrolysis, 32. D-arabinose, 33. Citrate utilization, 34. Malonate utilization, 35. Sorbose.

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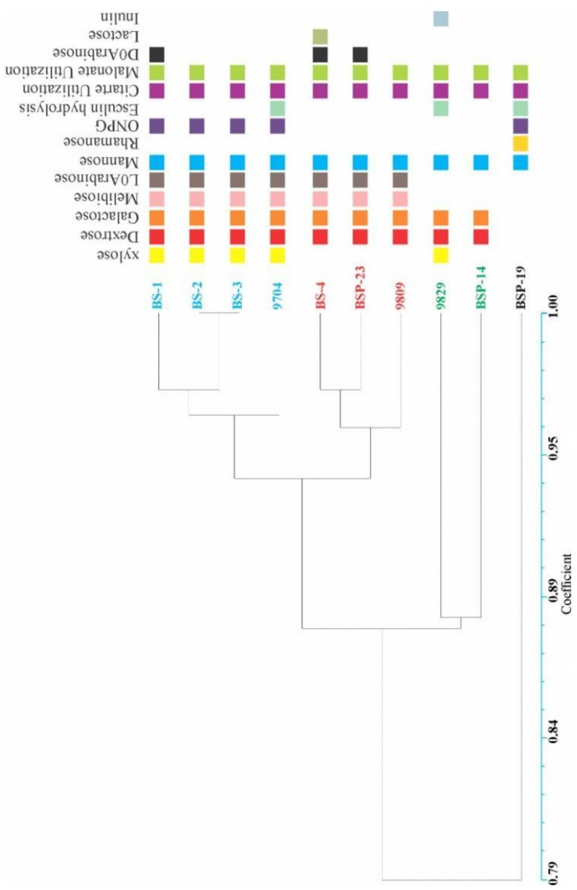


Plate: 4.4 Differential utilization of carbon sources by isolates of *Pseudomonas* as identified by Hi-carbohydrate™ kit test

Plate: 4.4 Differential utilization of carbon sources by isolates of Pseudomonas as identified by Hi-carbohydrate™ kit test

Bergey's Manual of Systematic Bacteriology, Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins).Comparison with selected nutritional and general phenotypic characteristics of the selected 10 isolates helped us to infer the putative species identity. A slight modification was done in the Standard data for selected nutritional and general phenotypic characteristics of selected fluorescent *Pseudomonas*. Wherever “d” was scored a +ve sign was assigned. Reactions obtained were scored as +ve or –ve. The relationship among the isolates was determined by converting +ve or –ve reactions into binary data (1 for present and 0 for absent). Similarity matrices were calculated using NTSYS computer program and cluster analysis was done by SHAN program using UPGMA method (Bergey, D. H., Hendricks, D., Holt, J. G., & Sneath, P. H. (1984); Bergey’s Manual of systematic bacteriology. Vol. 2. Williams and Wilkins).).

Analysis of 18 isolates (including selected fluorescent *Pseudomonas* species *(P. aeruginosa*, *P. fluorescence* bv. I, bv. II, bv. III, bv. IV, bv. V, *P. putida* biovar A, biovar B and 10 isolates used in the present investigation) of fluorescent *Pseudomonas* using 26 standard data for selected nutritional and general phenotypic characteristics of selected fluorescent *Pseudomonas*. Scored data of the 10 isolates were converted to the binary matrix as Positive as “1” or negative as “0”. The data was used to generate a similarity matrix using NTSYS (Numerical Taxonomy System Biostatistics) computer program. The similarity coefficient ranged from 0.58 to 1.00.

A perusal of dendrogram (Plate: 4.5) indicates that there was a major cluster consisting of 11 isolates, whereas 7 isolates *P. fluorescence* bv. I to V (standard isolates), *P. putida* bv. A and B (standard isolates) were found to be different from the rest of the isolates. The major cluster A and cluster B consisted of 11 and 7 isolates and shared 0.58 (Coefficient) / 58 % similarity. Major cluster A was further divided into sub-cluster A1 and A2 and consisted of 1, and 10 isolates respectively sharing about 0.70 (Coefficient) / 70 % similarity. The cluster A2 was further divided into sub-cluster A2(a) and A2(b) and consisted of 8, and 2 isolates respectively sharing about 0.74 (Coefficient) / 74% similarity. The cluster A2 (b) consisted of 2 isolates sharing about 0.81 (Coefficient)/ 81 % similarity.

The cluster A2 (a) was further divided into sub-cluster A2 (a1) and A2 (a2) and consisted of 6, and 2 isolates respectively sharing about 0.90 (Coefficient) / 90% similarity. The cluster A2 (a1) consisted of 6 isolates and were further subdivided into 2, A2 (a1-1) and A2 (a1-2) and consisted of 5 and 1 isolates sharing about 0.92 (Coefficient) / 92% similarity. Cluster analysis grouped the standard isolate *P. aeruginosa* and the 10 isolates (used in the present investigation) into cluster A and the rest of the other standard isolates into cluster B. Based on the standard data for selected nutritional and general phenotypic characteristics *P. aeruoginosa* expressed 70% similarity with the 10 isolates in the present investigation which indicated that the standard data set also derived the component of species identification. Based on the biochemical tests all the 10 isolates were identified as *P. aeruoginosa*.

4.2.8 Sequence analysis of fluorescent *Pseudomonas*.

Chromosomal 16S rRNA DNA sequences retrieved from different fluorescent *Pseudomonas* isolates as input for BLASTN. Chromosomal 16S rRNA DNA sequences of 10 isolates were queried using *Pseudomonas* Genome Database using the dialogue http://www.pseudomonas.com/ to search for similar sequences. Similar sequences of type strains were selected and retrieved in FASTA format. The sequences along with Chromosomal 16S rRNA DNA sequence were aligned using MUSCLE. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree inferred from 500 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the chromosomal 16S rRNA intervening DNA sequence analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

The percentage of replicate trees in which the associated chromosomal 16S rRNA intervening DNA sequence was clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). Initial tree(s) for the the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with

Table: 4.4 Standard data for selected nutritional and general phenotypic characteristics of selected fluorescent *Pseudomonas*.

S. No.	Properties	*P.aeruginosa*	*P. fluorescence* Biovar					*P. putida* biovar	
			I	II	III	IV	V	A	B
1	lactose	—	—	—	—	—	—	—	—
2	d-Xylose	—	+	d	d	d	d	d	d
3	maltose	—	—	—	—	—	—	—	—
4	d-Fructose	+	+	d	+	+	+	+	+
5	d-Glucose	+	+	+	+	+	+	+	+
6	d-Galactose	—	+	+	d	+	d	—	d
7	Trehalose	—	+	+	d	+	d	—	—
8	Sucrose	—	+	+	—	+	d	—	d
9	l-Arabinose	—	+	+	d	+	d	d	+
10	d-Mannose	—	+	+	+	+	d	d	d
11	inulin	—	—	—	—	—	—	—	—
12	glycerol	+	+	+	+	+	+	+	+
13	salicin	—	—	—	—	—	—	—	—
14	m-Inositol	—	d	+	d	+	d	—	—
15	Sorbitol	—	+	+	d	+	d	—	d
16	Mannitol	+	+	+	d	+	d	d	d
17	Adonitol	—	+	—	d	—	d	—	—
18	Erythritol	—	d	d	+	—	d	—	—
19	Cellobiose	—	—	—	—	—	—	—	—
20	d-Arabinose	—	—	—	—	—	—	—	—
21	citrate	+	+	+	+	+	+	+	+
22	Malonate	+	+	+	d	+	d	d	+
23	Gelatin liquefaction	+	+	+	+	+	+	—	—
24	Denitrification	+	—	+	+	+	—	—	—
25	Growth at4°C	—	+	+	+	+	+	d	+
26	Growth at 41°C	+	—	—	—	—	—	—	—

The scored data indicates:- 1) -, ≥90% of strains negative; 2) +, ≥90% of strains positive ; 3) d, 11±89% of strains positive.

70

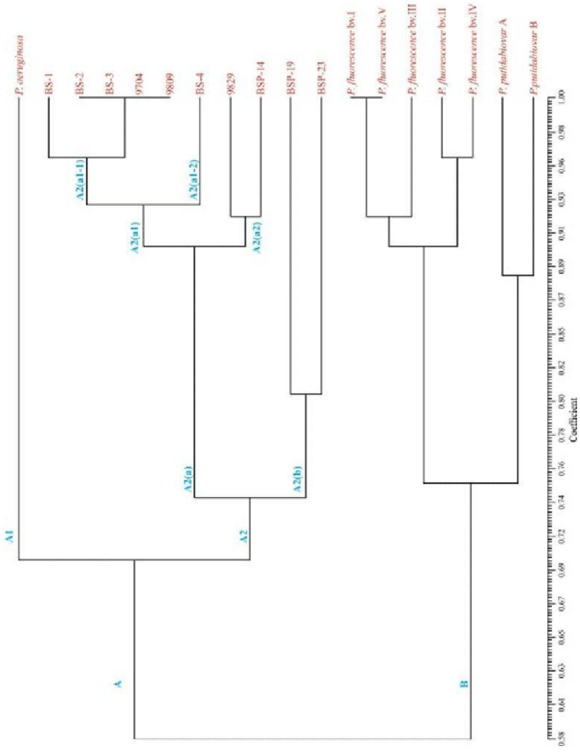


Plate: 4.5 Nutritional and general phenotypic characteristics of selected fluorescent *Pseudomonas*

Plate: 4.5 Nutritional and general phenotypic characteristics of selected fluorescent *Pseudomonas* superior log likelihood value. Evolutionary analyses were conducted in MEGA X (Kumar et al 2018). No. of nucleotide sequences involved in analysis, a tree with the highest log-likelihood, and the total number of positions in the final data set for each isolates analyzed for 16S rRNA DNA sequences are presented in Table: 4.5. The Maximum likelihood analysis of the chromosomal 16S rRNA intervening DNA sequences retrieved from different fluorescent *Pseudomonas* isolates revealed the presence of distinct phylogenetic groups (Plate: 4.5).

Based on the phylogenetic affinities of the 16S rRNA DNA sequence derived from the isolates **BS-1, BS-2, BS-4, 9704, 9829, 9809, and BSP 19** species identity is delineated as *Pseudomonas aeruginosa* and for BS-3 as *Pseudomonas chlororaphis* subsp. *Chlororaphis* and for BSP-14 and BSP 23 as *Pseudomonas mesoacidophila*. Based on reports of Stanier et al (1966) and Blazevic et al. (1973) some tests may help differentiate species of *Pseudomonas*. Blazevic et al. (1973) suggested that the test for sensitivity to kanamycin and resistance to carbenicillin and nitrate reduction would provide a means of identifying *P. fluorescens* and *P. putida* and separating them from *P.aeruginosa*.

Donna et al., 1973 differentiated *P. fluorescens, P. putida,* and *P. aeruginosa* by performing the antibiotic test, gelatine liquefaction, and nitrate reduction. The results obtained in this investigation are following the reports of Ramezanpour et al. (2010), differentiated *P. fluorescens, P. putida,* and *P. aeruginosa* based on biochemical characterization. Lemanceau et al. (1995) had used ten substrates that differentiated flax and tomato isolates. Most of the species designation done in the present investigation is as per Stanier et al (1966) keys.

4.2.9 Screening of fluorescent *Pseudomonas* isolates for phosphate solubilization

After nitrogen, Phosphorous is the most important plant growth-limiting nutrient. Phosphorous is available in soil abundantly in both organic and inorganic form (Khan et al. 2009). Soil regardless of the large reservoir of phosphorous, the amount of phosphorous available to plants is very low because the majority of soil phosphorous is found to be in insoluble forms or present as an inorganic mineral. such as apatite or in an organic form such as inositol phosphate (soil

Table: 4.5 16S rRNA Gene Sequence analysis of ten *Pseudomonas* isolates using 16S and 27F+1492R primer set

Code of the Query sequence	Species identity (Accession No.) of matching Sequence	% Identity	E-value
BS-1_16SF-GA 7542.ab1	*Pseudomonas aeruginosa* PAO1 (chromosome)(NC_002516)	98.696	0
BS-2_1492-R-GA-7542.ab1	*Pseudomonas aeruginosa* SP4527 (chromosome) (NZ_CP034409.1)	93.676	0
BS-3_16SF_GA_7542	*Pseudomonas chlororaphis* subsp. *chlororaphis* DSM 50083 (chromosome) (NZ_CP027712.1)	76.562	5.8E-09
BS-4-16S-F_GA-7542.ab1	*Pseudomonas aeruginosa* H25883 (chromosome) (NZ_CP033686.1)	99.259	0
9704_16SF_GA_7542	*Pseudomonas aeruginosa* BH9 (chromosome) (NZ_CP029713.1)	95.642	0
7542_**9829**-16SF	*Pseudomonas aeruginosa* isolate paerg004 (paerg004-contig000) (NZ_LR130531.1)	96.750	0
7542_**9809**_16SF_GA_7542	*Pseudomonas aeruginosa* PA34 (chromosome)(NZ_CP032552.1)	98.953	0
BSP-14_16SF_GA_7542	*Pseudomonas mesoacidophila* ATCC 31433 (chromosome 2) (NZ_CP020738)	86.580	0
BSP-19_16SF_GA_7542	*Pseudomonas aeruginosa* SP4527 (chromosome) (NZ_CP034409.1)	87.947	0
BSP-23_16SF_GA_7542	*Pseudomonas mesoacidophila* ATCC 31433 (chromosome 2) (NZ_CP020738)	86.254	0

Sequencing site: Eurofins laboratory Bangalore for Sanger’s sequencing
Alphabets in parenthesis represent gene bank accession number
E-value represents the number of hits

phytate) (Glick 2012) Plants absorb phosphorous only in two soluble forms, monobasic ($H_2PO^-_4$) and dibasic ($H_2PO^{2-}_4$) ions (Bhattacharyya and Jha 2012).In this context, microbes having phosphorous solubilizing activity may provide an available form of phosphorous to plants. The capacity to solubilize the fixed form of phosphate through the release of organic acids is one of the important features of fluorescent *Pseudomonas.* In present investigation all the *Pseudomonas* isolates had the potential to differentially utilizing tri-calcium phosphate and was qualitatively estimated on pokivskya agar media supplemented with bromocresol blue and quantitatively estimated in pikovskya broth medium.

4.2.9.1 Qualitative Screening of fluorescent *Pseudomonas* isolates for phosphate solubilization.

A qualitative assessment of *Pseudomonas* isolates for phosphate solubilization was done on pikovskya's agar medium. This agar medium was supplemented with Bromo cresol blue (pH indicator) before pouring into the Petri plate. Fresh cultures of *Pseudomonas* were spot inoculated in the center of the Petri plate. After inoculation plates were incubated for 48 hrs. *Pseudomonas* isolates having the potential to solubilize the phosphate in the Petri plate produce an orange color circular zone around the inoculated area by acidification (Plate: 4.6). *Pseudomonas* isolates having a higher potential to solubilize phosphate produces a large circular area. *Pseudomonas* isolates having phosphate solubilizing ability had categorized into high (+++), medium (++), and low (+). In present investigation 9704, BSP-19 and BSP-23 were found to be high phosphate solubilizer and categorized as high (+++). Isolates 9809 and BSP-14 were found to be medium phosphate solubilizers and categorized as medium (++). Whereas, isolates BS-1, BS-2, BS-3, BS-4, and 9829 were found to be low phosphate solubilizer and categorized as low (+) (Table: 4.6).

4.2.9.2 Quantitative estimation of fluorescent *Pseudomonas* isolates for Phosphate solubilization (liquid assay).

Quantitative estimation of phosphate solubilization of *Pseudomonas* isolates was done in pikovskya's broth. All the *Pseudomonas* isolates were inoculated in pikovskya broth and incubated for 7 days on a rotary shaker. The amount of inorganic phosphate solubilized by inoculated *Pseudomonas* isolates was estimated by preparing the standard curve at a wavelength of 610 nm. It was observed that all the ten isolates showed variation in the utilization of supplemented tri-calcium in the medium. The phosphate solubilization concentrations were expressed as µg/ml. In present investigation phosphate solubilisation varied from 108µg/ml to 357.5µg/ml (Table: 4.6). Highest phosphate solubilisation was observed for *Pseudomonas* isolate 9704 (357.5 ± 1.5 µg/ml) led to 69.7% increase over control followed by 9829 (65.8%), BSP-19 (48.5%), BSP-23 (47.5%), BSP-14 (45.4%), 9809 (44.4%), BS-4 (43.6%), BS-3 (42%), BS-2 (33.3%) and BS-1 (23.6%) increase over control.

Pseudomonas isolate 9704 was isolated from the Jagdalpur region of Bastar district and identified as *P. aeruginosa by* performing different biochemical tests. *In-vitro* analysis of 9704 revealed that it was also possessing good phosphate solubilizing ability among ten isolates. Phosphorous is responsible for plant growth and development and it is involved in several key functions like photosynthesis, the transformation of sugars, and starches in plant systems (Sultenfuss and Doyle 1999). Further PGP effects of *Pseudomonas* isolate 9704 will be proved by seed bacterization in chickpea, soybean, and wheat. (Paul and Sinha 2017) found that *Pseudomonas* strain KUPSB12 was effective in phosphate solubilization index 2.85 in pikovskya agar plates along with very high soluble phosphate production of 219 ± 0.33µg/ml in a liquid medium. Sharma et al. (2017) also found that potential phosphate solubilizing *Pseudomonas aeruginosa* strain An-Mg isolated from the rhizosphere soil of apple tree could be used as a biofertilizer as well as a potential biocontrol agent.

4.2.10 Quantitative analysis of fluorescent *Pseudomonas* isolates for IAA (Indole acetic acid) production.

Indole acetic acid is the important phytohormone that regulates plant root/shoot growth. It is reported that plant-associated bacteria possess the ability to synthesize and release IAA as a secondary metabolite and provide bacteria with a mechanism to influence plant growth (Patten and Glick 1996; Patten and Glick 2002). Tryptophan, identified as the main precursor for IAA biosynthesis and culture media supplemented with tryptophan increases IAA production (Zaidi et al. 2009; Spaepen and Vanderleyden 2011). In the present investigation screening of

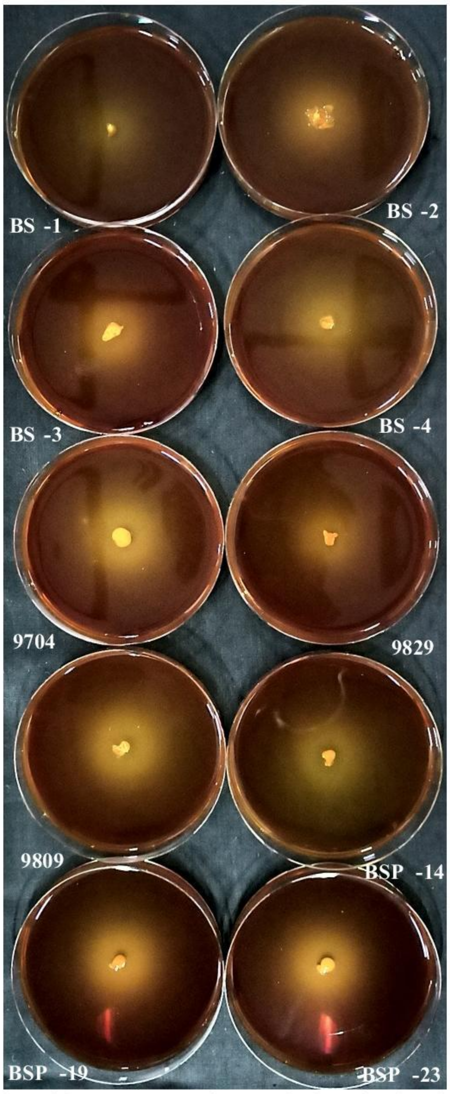


Plate: 4.6 Screening of fluorescent *Pseudomonas* isolates for phosphate solubilisation

Plate: 4.6 screening of fluorescent *Pseudomonas* isolates for phosphate solubilisation

Table: 4.6 Screening of fluorescent *Pseudomonas* isolates for Phosphate solubilisation

Isolates	Phosphate solubilisation in Pikovskya broth (µg/ml)	Plate assay for Phosphate solubilisation *
BS-1	$141.5^{G}\pm10.5$	+
BS-2	$162^{F}\pm7$	+
BS-3	$186.5^{E}\pm1.5$	+
BS-4	$191.5^{DE}\pm5.5$	+
9704	$357.5^{A}\pm1.5$	+++
9829	$316^{B}\pm5$	++
9809	$194.5^{CDE}\pm0.5$	+
BSP-14	$198.5^{CDE}\pm7.5$	++
BSP-19	$210^{C}\pm7$	+++
BSP-23	$206^{C}\pm4$	+++
CONTROL	$108^{H}\pm5$	-
C.D.	18.156	
SE(m)	5.764	
SE(d)	8.152	
C.V.	3.947	

* High phosphate solubiliser (+++)
Medium phosphate solubiliser (++)
Low phosphate solubilizer (+)

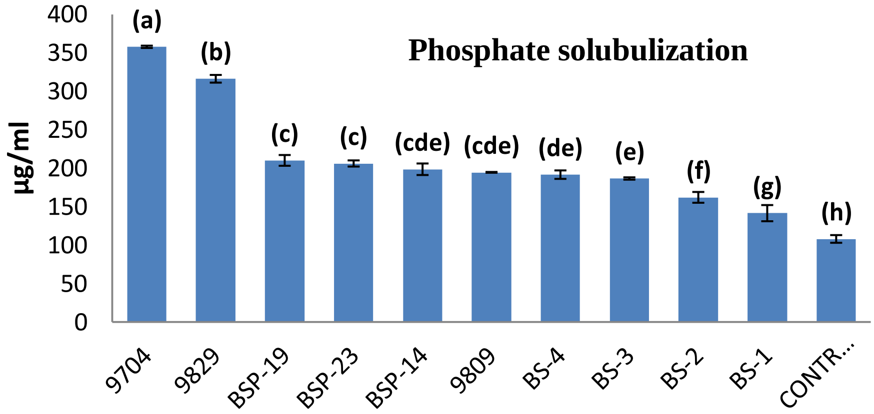


Figure: 4.1 Quantitative screening of fluorescent *Pseudomonas* isolates for Phosphate solubilization in Pikovskaya's broth.

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level
*Means followed by a common letter are not significantly different at the 5 % level
*Error bars indicate one standard error of the mean

ten *Pseudomonas* isolates for the production of IAA was done in DF minimal media supplemented with tryptophan (1.02g/L from 5mM stock solution). After incubation for 72 hrs, colorimetric readings were measured by absorbance at 535nm. The concentrations of IAA produced by 10 *Pseudomonas* isolates were evaluated by comparing it with a standard curve that was prepared by serial dilution (0-100µg/ml) using the commercial available IAA Quantity of IAA produced by ten *Pseudomonas* isolate ranged from 28.08 ± 0.085 µg/ml to 2.34±0.022 µg/ml (Table: 4.7). Among ten *Pseudomonas* isolates highest IAA was produced by isolate BSP-23 (28.08 ± 0.085 µg/ml) followed by BS-3 (24.29± 0.062µg/ml), 9809 (23.69±0.149 µg/ml), BSP-14 (13.75±0.041µg/ml), BS-4 (10.37±0.054 µg/ml), BSP-19 (8.32±0.068 µg/ml), 9829 (7.63±0.09 µg/ml), BS-1 (6.19±0.058 µg/ml), 9704 (3.29±0.02 µg/ml) and BS-2 (2.34±0.022 µg/ml).

Pseudomonas isolate BSP-23 was isolated from Bilaspur district soil samples and identified as *P. fluorescence by* performing different biochemical tests. *In-vitro* analysis among ten *Pseudomonas* isolates revealed that BSP-23 has produced more quantity (28.08 ± 0.085 µg/ml) of IAA. IAA is responsible for increasing root surface area and root length and thereby provides the plants greater access to soil nutrients. Further PGP effects of isolate BSP-23 were evaluated in the field. *Pseudomonas* Isolates producing IAA have a stimulatory effect on plant growth and increase N, P, K, Ca, and Mg uptake (Farzana and Radizah 2005). Sivasakthivelan and Saranraj (2013) analyzed that biocontrol strains *P. fluorescence* for IAA biosynthesis and studied the effect of its consequent manipulation on its PGP potential.

4.2.11 Screening of ACC deaminase containing fluorescent *Pseudomonas* isolates.

Generally, ethylene is a plant hormone essential for plant growth and development (Khalid et al. 2006). Ethylene is produced endogenously by all plants in response to different stresses. Apart from being a plant growth regulator ethylene is also been used as a stress hormone. Under stress conditions, endogenous levels of ethylene increase and lead to effect overall plant growth (Saleem et al. 2007; Bhattacharyya and Jha 2012).

Table: 4.7 Concentrations of IAA produced by fluorescent *Pseudomonas* isolates.

S. no	**Isolates**	**IAA production conc. (µg/ml)**
1	BS-1	6.19^{DE}±0.058
2	BS-2	2.34^{F}±0.022
3	BS-3	24.29^{B} ± 0.062
4	BS-4	10.37^{BCD}±0.054
5	9704	3.29^{E}±0.02
6	9829	7.63^{CDE}±0.09
7	9809	23.69^{AB}±0.149
8	BSP-14	13.75^{ABC}±0.041
9	BSP-19	8.32^{BCD}±0.068
10	BSP-23	28.08^{A} ± 0.085
	C.D.	0.236
	SE(m)	0.074
	SE(d)	0.105
	C.V.	23.611

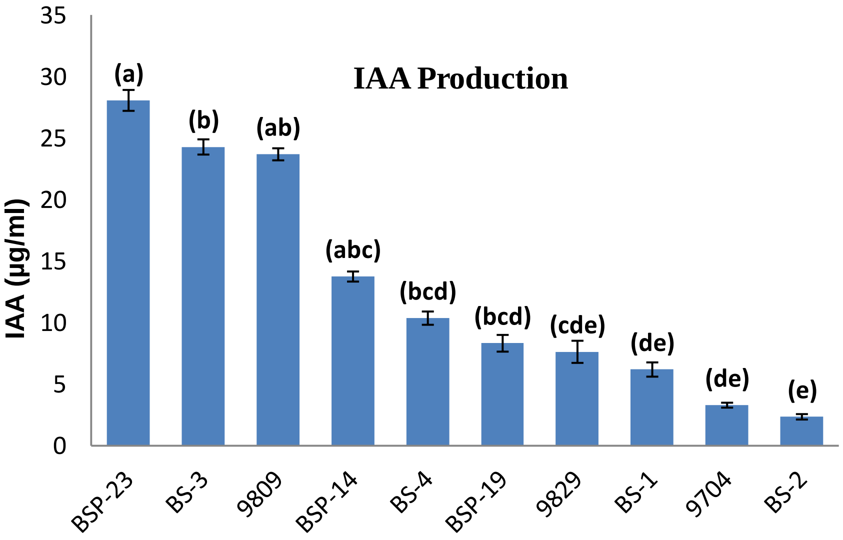


Figure: 4.2 Quantitative analysis of fluorescent *Pseudomonas* isolates for IAA (Indole acetic acid) production

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level
*Means followed by a common letter are not significantly different at the 5 % level
*Error bars indicate one standard error of the mean

Microbes that possess the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, facilitate plant growth by decreasing ethylene levels (Nadeem et al. 2007). Rhizobacteria utilize ethylene precursor ACC and convert it into 2 – oxobutanoate and NH_3 (Arshad et al. 2007)

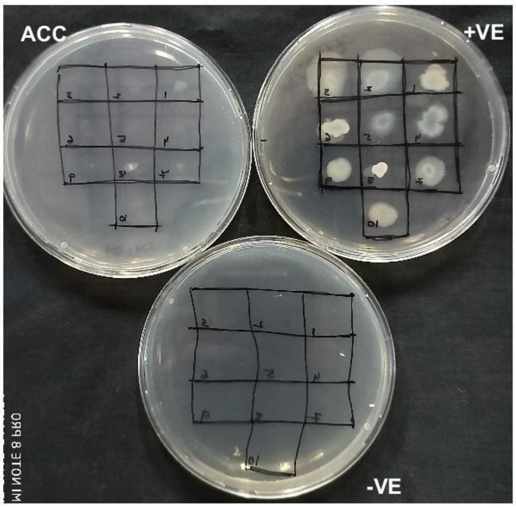
In the present investigation qualitative analysis of *Pseudomonas* isolates for finding the ability to utilize the ACC deaminase was done in DF salt minimal agar media supplemented with ACC. In this media ACC was the sole source of nitrogen, plates containing only $(NH_4)_2SO_4$ served as a positive control, and plates containing only DF minimal media without $(NH_4)_2SO_4$ and ACC serve as a negative control. All *Pseudomonas* isolates showed positive control (Plate: 4.7), no growth on negative control and among 10 isolates five isolates showed growth in plates which is supplemented with ACC. *Pseudomonas* isolates BS-1, BS-4, 9809. BSP-14 and BSP-19 had utilized ACC deaminase and degraded the ACC.

Pseudomonas isolates BS-1, BS-4, 9809, BSP-14, and BSP-19 found to produce IAA, phosphate solubilization, and also tend to produce ACC deaminase enzyme which decreases the level of ethylene in the plant by utilizing ACC which is the precursor for ethylene and reduces stress in plants. Many reports say that IAA and ACC could work together and promote plant growth, especially root elongation (Glick et al. 1998; Glick 2014). The major effect of seed inoculation with ACC producing rhizobacteria is plant root elongation, enhancement of rhizobial nodulation, and N, P, and K uptake (Nadeem et al. 2007). Magnucka and Pietr 2015 showed that the highest activity of the ACC enzyme exhibited by *Pseudomonas* isolates PO366 (wheat rhizosphere), PO283 (wheat rhizosphere), and RZ310 (rape rhizosphere) were 3.70, 4.32, and 16.50 mmol α-KB/g protein/h respectively in DF minimal media.

4.2.12 Screening and characterization of potential *Pseudomonas* isolates for siderophore production

Generally, in aerobic environment iron occur as Fe^{3+} in soil and it forms hydroxide and oxyhydroxides which is an insoluble and inaccessible form of iron both to plants and microorganisms (Rajkumar et al. 2010). Microorganism having the ability to produce low molecular mass iron chelators referred to as siderophores which have high association constant for complexing iron can acquire iron and

1. BS-1, 2. BS-2, 3.BS-3, 4. BS, 5. 9704,
6. 9829, 7. 9809, 8. BSP-14 9. BSP-19 10. BSP-23



+ve control ($(NH_4)_2SO_4$),
-ve control (without ACC),
With only ACC as sole source of nitrogen

Plate: 4.7 Screening for 1- Aminocyclopropane-1-carboxylic acid deaminase (ACC Deaminse) containing Fluorescent *Pseudomonas* isolates.

Plate: 4.7 Screening for 1- Aminocyclopropane-1-carboxylic acid deaminase (ACC Deaminase) containing Fluorescent *Pseudomonas* isolates.

form Fe^{3+}- siderophores complex on the bacterial membrane and reduced Fe^{3+} to Fe^{2+} which is further released into the cell from the siderophore through a gating mechanism linking inner and outer membranes. Plants uptake iron from bacterial Fe^{3+}- siderophores complexes by ligand exchange reaction (Schmidt 1999).

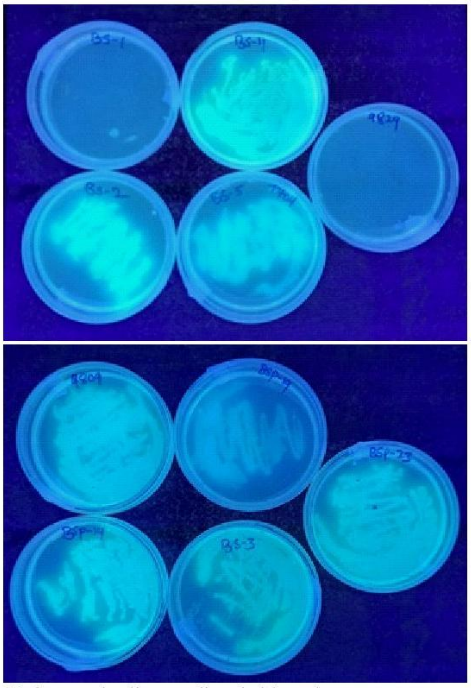
4.2.12.1 Qualitative estimation of siderophore production

Qualitative screening for siderophore production by ten *Pseudomonas* isolates was done by the hydroxyquinoline test. In the hydroxyquinoline test, Kings B agar media was supplemented with strong iron-chelating agent 8-hydroxyquinoline. Then Kings B agar media was inoculated with *Pseudomonas* isolates and incubated for 48hr. 8-hydroxyquinoline is a strong iron chelator and it lowers the amount of available iron in the agar media. Only those isolates will grow in such media which produces strong iron-chelating siderophore. In the present investigation, all *Pseudomonas* isolates except BS-1 and 9829 were able to grow in medium (Plate: 4.8).

4.2.12.2 Quantitative estimation of siderophore production

Quantitative estimation of siderophore production by *Pseudomonas* isolates was done by CAS assay (chrome asurol assay). Using CAS assay percent siderophore unit was determined by spectrophotometer. *Pseudomonas* isolate BSP-19 (79.71%) had produced the highest amount of siderophores followed by BS-4 (56.3%), BS-1 (54.82%), BSP-14 (50.66%), BS-3 (50.3%), 9829 (50.25%), 9704 (48.75%), BS-2 (46.78%), 9809 (40.25%) and BSP-23 (22.1%) (Table: 4.8).

Pseudomonas isolate BSP-19 was isolated from Bilaspur district soil samples and identified as *P. fluorescence* by performing different biochemical tests. *In-vitro* analysis among ten *Pseudomonas* isolates revealed that BSP-19 has produced the highest siderophore units (79.71%). Microbes produce siderophores for increasing the amount of soluble iron uptake. This increased siderophore production profits the plants and then selected these isolates for enhancing the iron availability and increase plant growth (Carvalhais et al., 2013, Hartmann et al., 2009). The rhizosphere as a plant nutrient-enriched site is a highly competitive environment for microbes. Some potential microbes are capable of producing some secondary metabolites which compete with other competitors which have the same niche and those metabolites include mainly the siderophores with antibiotics and



Hydroxyquinoline mediated siderophore test

Plate: 4.8 Screening of fluorescent *Pseudomonas* isolates for siderophores production

Plate: 4.8 Screening of fluorescent *Pseudomonas* isolates for siderophores production.

Table: 4.8 Quantitative and qualitative estimation of siderophore production by *Pseudomonas* isolates.

Isolate	% siderophore	Hydroxyquinoline*
BS-1	54.82^{CD}±0.15	Negative
BS-2	46.78^{BC}±0.028	Positive
BS-3	50.3^{CD}±0.04	positive
BS-4	56.3^{B}±0.005	positive
9704	48.75^{BCD}±0.023	positive
9829	50.25^{CD}±0.04	Negative
9809	40.25^{D}±0.082	positive
BSP-14	50.66^{CD}±0.15	positive
BSP-19	79.71^{A}±0.03	positive
BSP-23	22.1^{E}±0.01	positive
C.D.	0.253	
SE(m)	0.079	
SE(d)	0.112	
C.V.	7.623	

*Positive: Growth in Kings B media supplemented with 8-hydroxyquinoline
Negative: No growth in Kings B media supplemented with 8 hydroxyquinoline

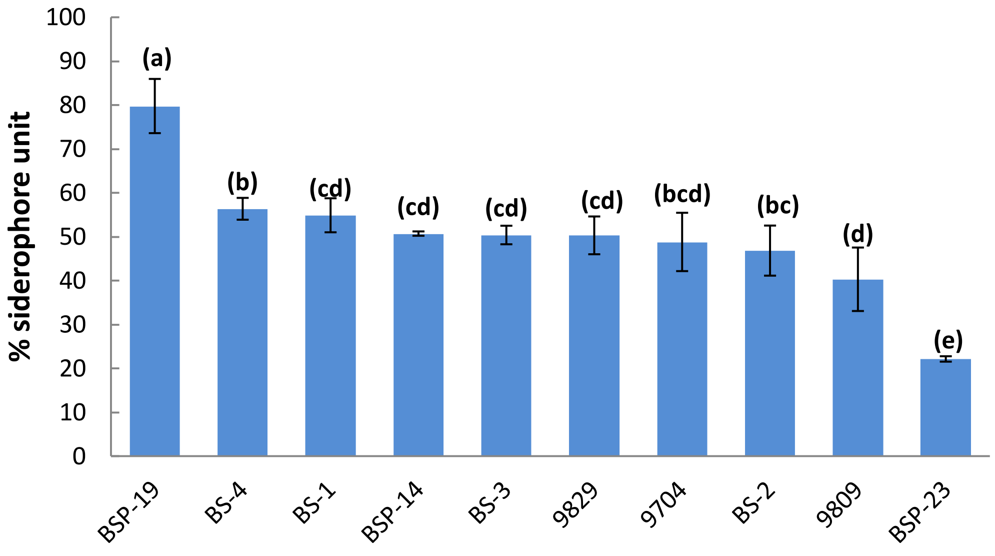


Figure: 4.3 Quantitative analysis of *Pseudomonas* isolates for siderophore production

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level
*Means followed by a common letter are not significantly different at 5 % level
*Error bars indicate one standard error of the mean

toxins (Bais et al., 2006). Numerous studies of the plant growth promotion vis-a` -vis siderophore-mediated Fe-uptake as a result of siderophore producing rhizobacterial inoculations have been reported (Rajkumar *et al.,* 2010). The mechanism behind plant growth promotion and disease control by PGPR is due to antagonistic interaction by siderophore producing PGPR which results in the exclusion of pathogens in the rhizosphere by creating iron starvation (Leong, 1986; Scher, 1982). *Pseudomonas* is a potential biocontrol agent and produces siderophore that sequesters iron in the root environment, making it less available to competing for deleterious microflora (Kloepper *et al.*, 1980; Bagnasco *et al.*, 1998; Deshwal *et al.*, 2012).

4.3 *In-vitro* antagonistic activity by fluorescent *Pseudomonas* against *Macrophomina phaseolina*

The efficacy of the biocontrol activity of ten *Pseudomonas* isolates was studied against fungal pathogen *Maacrophomina phaseolina* following the confrontation assay technique (Kotasthane et al. 2017). There were differences in antagonistic abilities of ten *Pseudomonas* isolates against *Maacrophomina phaseolina* (Plate: 4.9). All the ten *Pseudomonas* isolates showed different degrees of growth inhibition of *M. phaseolina* ranging from 47.2% to 61.1% (Table: 4.9). Confrontation assays revealed *Pseudomonas* isolate 9704 and 9809 as potential antagonists against *M. phaseolina* with 61.1% inhibition followed by BSP-14 and BSP-23 with 60.5% inhibition, BS-1(60% inhibition), 9829 (59.4% inhibition), BSP-19 (60% inhibition), BS-4 (54.4% inhibition), BS-3 (50% inhibition) and BS-2 (47.2% inhibition).

Among ten *Pseudomonas* isolates 9704 which was found to be distinguished as *P. aeruginosa* by performing different biochemical tests and sequence analysis. 9704 is also a good phosphate solubilizer. Besides, in the present investigation confrontation assays revealed isolate 9704 as a potential antagonist against *M. phaseolina* with 61.1% inhibition. *Pseudomonas* spp. produce antibiotic DAPG (2,4 diacetylphologlucinol) is a major determinant of biological control of soil born plant pathogens (Thomashow and Weller 1996). Similar results were also obtained by Kotasthane et al (2017) reported fluorescent *Pseudomonas* isolates P66, P141, P144, P166, and P174 were antagonists against *Rhizoctonia solani* and *Sclerotium rolfsii* following confrontation assays.

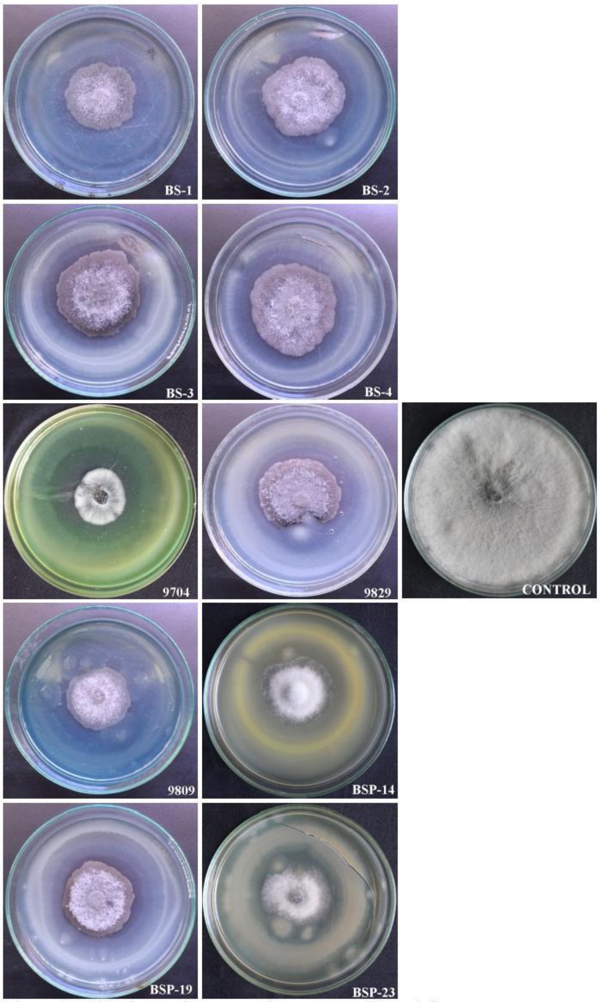


Plate: 4.9 Confrontation assay between collected fluorescent *Pseudomonas* and *Rhizoctonia bataticola*

Plate: 4.9 Confrontation assay between collected fluorescent *Pseudomonas* and *Rhizoctonia bataticola*

Table: 4.9 *In-vitro* antagonistic activity by fluorescent *Pseudomonas* against *Macrophomina phaseolina* (72 hr after incubation).

S. No	Isolate	Inhibition %
1	BS-1	60.00
2	BS-2	47.20
3	BS-3	50.00
4	BS-4	54.44
5	9704	61.10
6	9829	59.40
7	9809	61.10
8	BSP-14	60.50
9	BSP-19	60.00
10	BSP-23	60.50

4.3. 1 *In-vitro* infection model for *Macrophomina phaseolina* causing soybean charcoal rot

In the present investigation used plastic Petri plates slightly modified by cutting one edge in an arch. The lead and bottom of plastic Petri plates were fixed in position by sealing the sides with transparent sticky tapes. From the exposed edge of the cut side (arch), the soil was filled in the Petri plate cavity. The seed was directly sown in the Petri plates filled with soil (Plate: 4.10). No pre-germinated seed is required. Soybean seeds were sown in several Petri plates and placed in a vertical position in a tray and incubated in an incubator fitted with fluorescent light (figure: 1A and B). Uniform germination of the soybean seed in Petri plates was observed in three days. The root zone can be exposed by slowly removing the lid of Petri plates (figure: 1D). The technique allowed us for the easy of handling the germinated seedling in the Petri plates under the stereo binocular microscope (figure: 1C). Access to the root zone without disturbing the plant or root is possible and therefore one can remove root or part of root from the soil for further analysis. This technique also allowed us to perform bipartite (plant roots x pathogen) and tripartite (plant roots x pathogen x *Pseudomonas*) interactions under lab conditions.

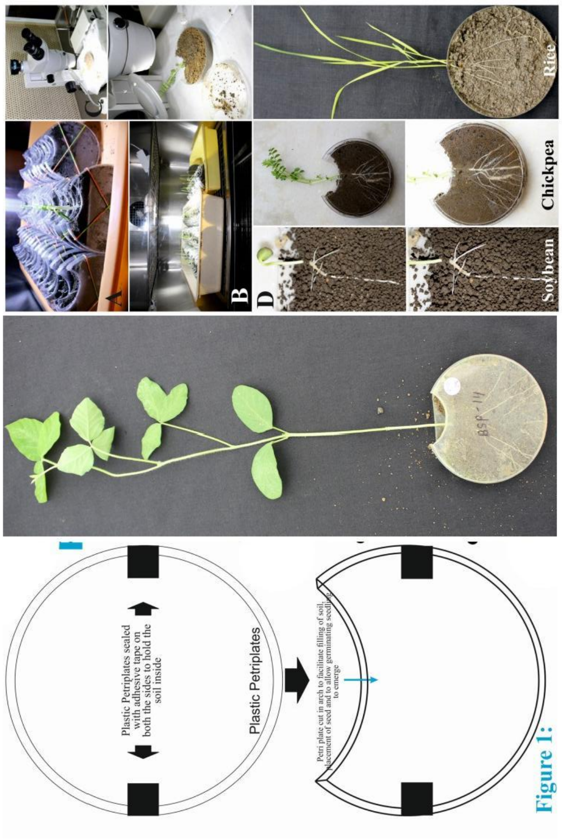


Plate: 4.10 *In-vitro* infection model for *Macrophomina phaseolina* causing soybean charcoal rot. Figure:1 schematic representation of petri plate assay model; Figure: 1A & B Petri plates placed in vertical position in a tray and incubated in an incubator fitted with fluorescent light; Figure: 1C handling the petriplates under the stereo binocular microscope Figure:1D Technique showing exposed roots of different seedlings

Plate: 4.10 In-vitro infection model for Macrophomina phaseolina causing soybean charcoal rot. Figure: 1 schematic representation of petri plate assay model; Figure: 1A & B Petri plates placed in vertical position in a tray and incubated in an incubator fitted with fluorescent light; Figure: 1C handling the petriplates under the stereo binocular microscope Figure:1D Technique showing exposed roots of different seedlings

4.3.2 Bipartite interaction (Soybean root x *M. phaseolina*)

Microsclerotia inoculated near the roots (A) started germinating and the advancing mycelia threads (3DAI) was able to stick on the root surface (B) was observed that on the root surface several mycelial threads are attached. Once adhered to the surface of roots the mycelial growth of *M. phaseolina* starts colonizing the roots. In the advanced stage of mycelia colonization on the root surfaces, the tissue gets killed and becomes dark and shrinks at a later stage (C). On the lower part of stem roots and split portion of the stem and roots due to the profuse formation of microsclerotia was observed (20DAI) (H and I). It was also observed that plants that died due to charcoal rot retained leaves attached to the petiole (Plate: 4.11).

4.3.3 Tripartite interaction (soybean roots x *M. phaseolina* x bioagent)

Confrontation assays were performed against *M. phaseolina* and isolate of fluorescent *Pseudomonas* 9704 (J) which resulted in 61.1% inhibition. Based on the antagonistic behavior of *Pseudomonas* isolate 9704 was selected for tripartite interaction in the proposed plate assay technique. Soybean seedling was derived after seed treatment with isolate 9704 and root imprinting was done on Kings' B media (K). The root imprinting technique provides the evidence for root colonization of treated seed with fluorescent *Pseudomonas* and no colonization in roots not treated with fluorescent *Pseudomonas* (Plate: 4.12).

Microsclerotia were inoculated near roots of young growing seedling (seedlings derived after seed treatment with isolate 9704 along with untreated control). A noticeable difference was observed for microsclerotia germination (K) on roots which were colonized by fluorescent *Pseudomonas* as compared to uncolonized roots. Microsclerotia of *M. phaseolina* on roots colonized with *Pseudomonas* isolate 9704, germinated very late (4DAI). Mycelia attachment, growth, and microsclerotia formation were confined to outer root layers even after 20 days of inoculation (M and N). It was therefore speculated on the roots antifungal metabolite might have released by colonized fluorescent *Pseudomonas* isolate 9704 (2, 4 Diacetylpholoroglucinol) which might have confined the mycelia growth and microsclerotia formation of inoculated *M. phaseolina* in the outer layers of roots. Bressano et al (2010) described a new *in vitro* method to infect

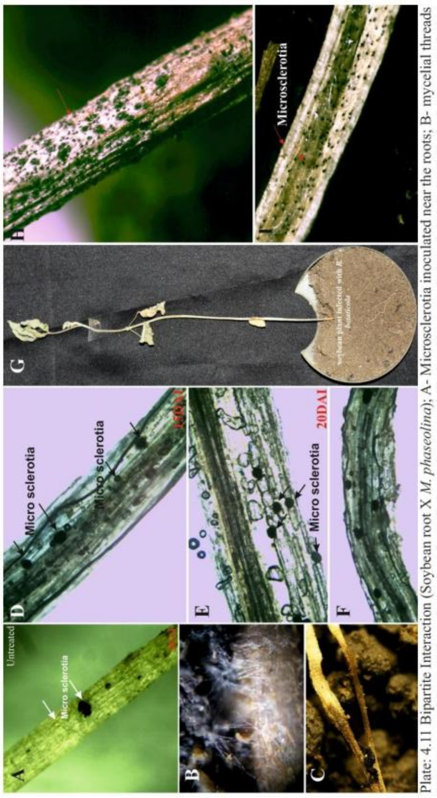


Plate: 4.11 Bipartite Interaction (Soybean root X *M. phaseolina*); A- Microsclerotia inoculated near the roots; B- mycelial threads (3DAI) were able to stick on the root surface; C- Root tissue gets killed and become dark and shrink; D, E, F- the lower part of stem roots and G, H and I-splitted portion of the stem due profuse formation of microsclerotia.

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plate: 4.11 Bipartite Interaction (Soybean root X M. phaseolina); A- Microsclerotia inoculated near the roots; B- mycelial threads (3DAI) were able to stick on the root surface; C- Root tissue gets killed and become dark and shrink; D, E, F- the lower part of stem roots and G, H and I-split portion of the stem due to profuse formation of microsclerotia.

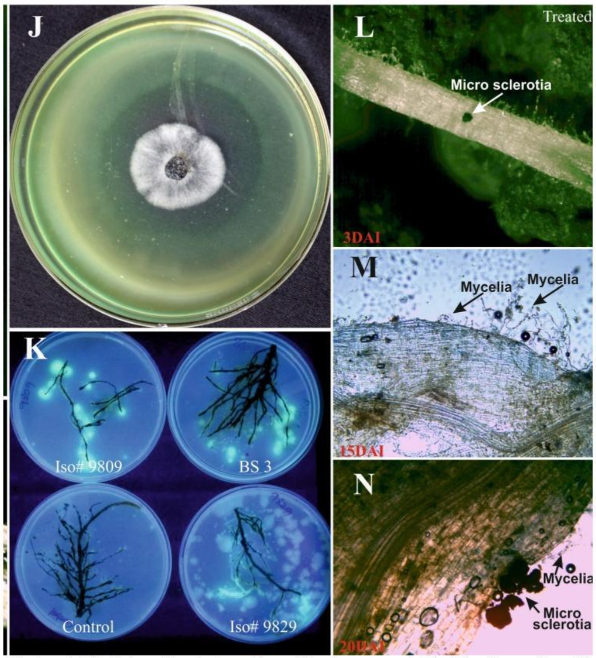


Plate: 4.12 Tripartite interaction (Soybean root X *M. phaseolina* X *Pseudomonas* spp); J- Confrontation assay against *M. phaseolina*; K- Root imprinting on Kings' B media; M & N Mycelia attachment, growth and microsclerotia formation was confined to outer root layers even after 20 days of inoculation

Plate: 4.12 Tripartite interaction (Soybean root X M. phaseolina X Pseudomonas spp); J- Confrontation assay against M. phaseolina; K- Root imprinting on Kings' B media; M & N Mycelia attachment, growth, and microsclerotia formation was confined to outer root layers even after 20 days of inoculation

plants with soil born pathogen *M. phaseolina* that may apply to the examination of the prepenetration and penetration phases of other soil born fungi as well as the early responses of the host plant. Chakraborty et al (2011) reported pre-penetration and penetration including microsclerotia germination, hyphal penetration, and pathogen colonization into the infected root tissue of two soybean cultivars, Williams (susceptible) and Hadgeston (resistant), with reported differences in the level of resistance to *M. phaseolina*, were investigated by histological analysis.

4.4 Effect of seed bacterization with *Pseudomonas* isolates on the germination and growth of soybean seedlings

Seed germination is the major issue in soybean. Poor germination or failure in germination may be due to poor quality of seed, unavailability of sufficient moisture in the soil and many other biotic and abiotic factors acts as a hindrance to germination (Links et al. 2020). Many studies state that seed inoculation with plant growth-promoting rhizobacteria (PGPR) before sowing will enhance germination and seedling establishment (Anitha et al. 2013; Chakraborty et al. 2011).

In present investigation effect of seed bacterization with ten *Pseudomonas* isolates on the germination and growth of soybean seedlings were studied. In comparison with the untreated control, all the *Pseudomonas* isolates significantly enhanced both germination and growth of soybean (CG SOYA-1) seedlings on 3rd 4th and 7th day (Plate: 4.13). Earlier germination of soybean seeds treated with *Pseudomonas* isolates was recorded (Plate: 4.13a). Besides, highest root (21.3 ± 0.30 cm) and shoot (14.05 ± 0.48 cm) growth was recorded on 7th day in soybean seeds treated with isolate 9704 (Table: 4.10) which led to 63.61% and 13.02% increase over control respectively (Plate: 4.13c). Significant increase in root length was observed with isolates 9809 (61.72%) > BSP-23 (61.05%) > 9829 (58.10%) > BSP-14 (58.10%) > BS-4 (55.45%) > BSP-19 (52.30%) > BS-1(51.41%) >BS-2 (49.83%) > BS-3 (33.18%) over control in respective order. Similarly significant increase in shoot length was observed with isolates 9809 (13.02%) > BSP-23 (11.6%) > 9829 (7.34%) > BS-2 (6.58%) > BSP-14 (6.58%) > BS-4 (3.4%) over control in respective order. Whereas *Pseudomonas* isolates BS-1, BS-3 and BSP-19 showed a decrease in shoot growth over control (Figure: 4.4). The results suggest that *Pseudomonas* isolate 9704 is a plant growth-promoting rhizobacteria

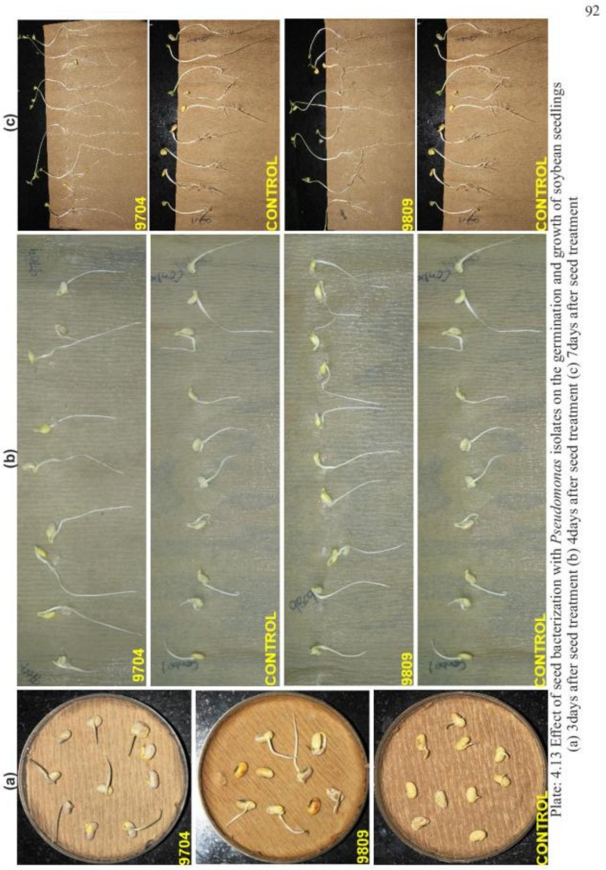


Plate: 4.13 Effect of seed bacterization with *Pseudomonas* isolates on the germination and growth of soybean seedlings (a) 3days after seed treatment (b) 4days after seed treatment (c) 7days after seed treatment

Plate: 4.13 Effect of seed bacterization with *Pseudomonas* isolates on the germination and growth of soybean seedlings (a) 3days after seed treatment (b) 4days (c) 7days after seed treatment.

Table: 4.10 Effect of seed bacterization with *Pseudomonas* isolates on the germination and growth of soybean seedlings (seven days after seed treatment)

S.NO	Isolates	Shoot length (cm)	Root length (cm)
1	9829	12.25^{ABC} ± 0.58 (7.34 %)	18.5^{ABC}±1.19 (58.10%)
2	9704	14.05 ^{A} ± 0.48 (19.2%)	21.3^{A}±0.30 (63.61%)
3	9809	13.05^{AB} ± 0.63 (13.02%)	20.25^{AB}±0.58 (61.72%)
4	BS-1	10.75^{BCD} ± 0.32 (**)	15.95^{C}±0.83 (51.41%)
5	BS-2	12.15^{ABCD} ± 0.40 (6.58%)	15.45^{C}±0.96 (49.83%)
6	BS-3	8.5 ^{CDE}± 0.29 (**)	11.6^{D}±0.74 (33.18%)
7	BS-4	11.75^{ABCD} ± 0.47 (3.4%)	17.4^{BC}±0.90 (55.45%)
8	BSP-14	12.15^{ABCD} ± 0.78 (6.58%)	18.5^{ABC}±0.96 (58.10%)
9	BSP-19	9.8^{DE} ± 0.42 (**)	16.25^{C}±0.87 (52.30%)
10	BSP-23	12.85^{ABC} ± 0.52 (11.6%)	19.9^{AB}±0.40 (61.05%)
11	CONTROL	11.35^{E} ± 0.31	7.75^{E}±0.40
	C.D	1.39	2.22
	SE(M)	0.49	0.79
	SE(D)	0.70	1.11
	C.V	13.75	15.02

Values in the parenthesis represent percentage increase over control
(**) = represent percentage decrease over control

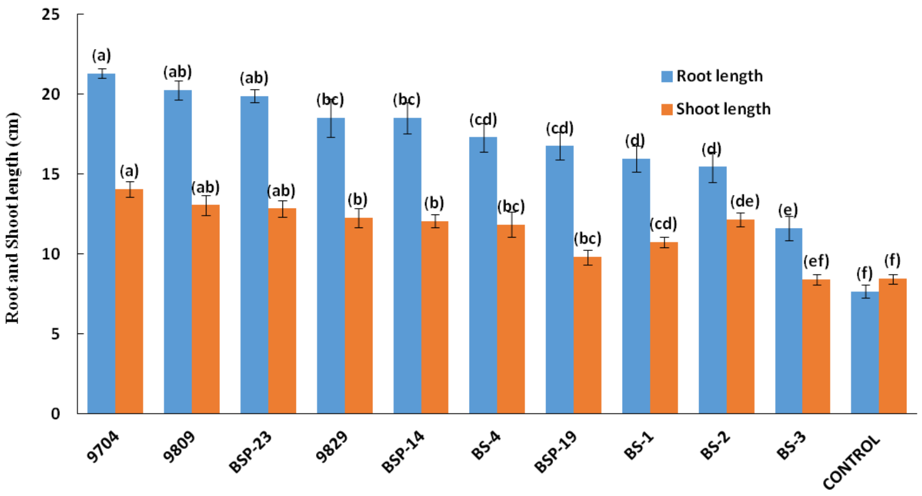


Figure: 4.4 Effect of seed bacterization with *Pseudomonas* isolates on the root shoot growth of soybean seedlings (seven days after seed treatment)

Means followed by a common letter are not significantly different at the 5 % level
Error bars indicate one standard error of the mean
All the treatment values are average of three replications

which have the potential for use in seed bacterization for higher germination and growth of soybean seedling. This phenomenon might be attributed to the production of auxins, lower levels of ethylene, or mineralization of nutrients by *Pseudomonas* isolate. Seed inoculated with PGPR showed an increase in the number and length of lateral roots and stimulates root hair elongation (Dobbelaere et al. 1999). Similar results were also obtained by Kumar and Dube (1992) fluorescent *Pseudomonas* RBT 13, isolated from tomato rhizoplane, resulted in increased seed germination (in terms of root and shoot length and weight) and yield of plants.

4.5 Plant growth-promoting response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolates

Fluorescent *Pseudomonas* is a well-known PGPR and improves plant growth and yield by a wide variety of mechanisms, including the production of siderophore, phosphate solubilization, production of plant growth hormones, enhancement of mineral uptake, and release of enzymes that regulates levels of ethylene hormones in the plant (Glick 1995). In the present investigation, ten *Pseudomonas* isolates were evaluated for their plant growth promotion ability and yield attributing character following seed treatment in soybean.

4.5.1 Pot experiment

Pot experiments were carried out at rainout shelter, College of Agriculture, I. G. K. V, Raipur during *Kharif-2019* to study the effect of *Pseudomonas* isolate on the growth and yield of soybean. The plant growth-promoting ability of ten *Pseudomonas* isolates was evaluated in soybean cultivar (CG SOYA-1) following seed treatment. Ten seeds of each treatment were sowed in each pot and three replications were maintained for each treatment. Observations were recorded after 110 days of sowing (Table: 4.11). Most of the studied traits such as shoots length, root length, number of branches, and number of filled pods were significantly different at the 5 % probability level. Pot experiment revealed that shoot length was highly stimulated by seed inoculation with *Pseudomonas* isolates and varied from 72.66±1.76 cm to 54.00±1.76 cm. The highest shoot length (72.66±1.76 cm), number of filled pods (83.66±8.95), and branches (7.00±0.00) were recorded in soybean plants treated with *Pseudomonas* isolate 9704 (Plate: 4.14). *Pseudomonas*

Plate: 4.14 PGP response in soybean (CG SOYA-1) following seed bacterization with flourescent *Pseudomonas* isolate 9704 under pot conditions

Plate: 4.15 PGP response in soybean (CG SOYA-1) following seed bacterization with flourescent *Pseudomonas* isolate BS-2 under pot conditions

Plate: 4.14 & 4.15 PGP response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolate 9704 BS-2 under pot conditions

Plate: 4.16 PGP response in soybean (CG SOYA-1) following seed bacterization with flourescent *Pseudomonas* isolate 9829 under pot conditions

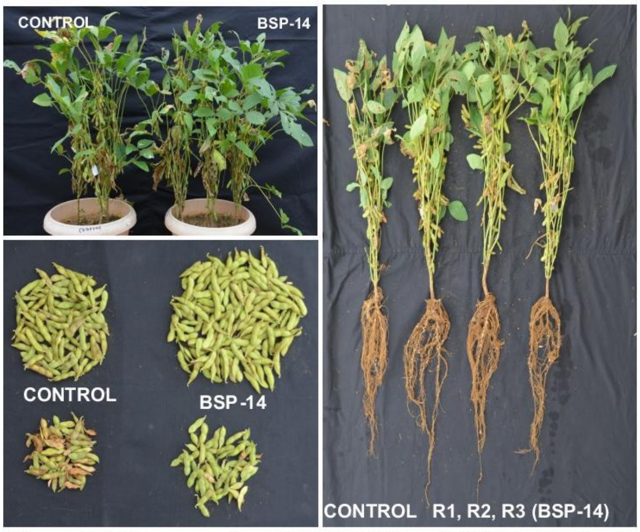


Plate: 4.17 PGP response in soybean (CG SOYA-1) following seed bacterization with flourescent *Pseudomonas* isolate BSP-14 under pot conditions

Plate: 4.16 & 4.17 PGP response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolate 9829 BSP-14 under pot conditions.

isolates BS-3 (59.00±5.68cm) recorded the highest root length. Whereas, *Pseudomonas* isolates BS-3, 9829, BSP-19, and BS-4 recoded the lowest shoot length over control. The highest percentage (82.83%) of filled pods was recorded with isolate 9704 (Plate: 4.14).

4.5.2 Field experiment

Field experiments were carried out at research cum instructional farm, College of Agriculture, I. G. K. V, Raipur during *Kharif-2019* to study the effect of *Pseudomonas* isolate on the growth and yield of soybean. Based on the results of variance analysis of soybean field data traits like shoot length, number of branches, filled pods, bundle weight, strawweight, and grain yield were significantly different at 5 % probability level. In a field experiment, shoot length, number of pods, bundle weight, strawweight, and grain yield parameters in soybean are influenced by ten *Pseudomonas* isolate treatments presented in Table: 4.12. A significant increase in shoot length was observed in all treatments over control. The maximum shoot length was observed in soybean plants treated with *Pseudomonas* isolate 9704 (88±3.23cm), which led to a 10% increase in shoot length over control, followed by 9.3%, 8.75%, 8.7%, 8.1%, 7.9%, 7%, 4.57% in BS-1, BSP-14, 9829, BS-3, BS-4, BS-2, and BSP-23 respectively. Whereas isolates BSP-19 and 9809 showed decreased shoot length percent over control. Seed treatment with *Pseudomonas* isolate had significantly affected filled pods. A significant increase in filled pods was recorded in soybean plants treated with *Pseudomonas* isolates over untreated control. Highest number of filled pods was seen in soybean plant treated with isolate 9704 (93.4±2.78), which led to 47.7% increase in filled pods over control, followed by BSP-23 (46.49%), BSP-19 (46.14%), BS-1 (45.54%), BS-3 (44.67%), BS-2 (36.29%), BS-4 (35.96%), BSP-14 (34.41%), 9829 (30.29%) and 9809 (28.65%).

Bundle weight, straw yield, and grain yield in soybean plants treated with *Pseudomonas* isolates were markedly increased in comparison to untreated or control (Figure: 4.5). The highest bundle weight (6891.67 kg/ha) and straw yield (5466.67 kg/ha) were recorded in soybean plants treated with *Pseudomonas* isolate BSP-23 led to 30.35 % and 34.14% increase over control respectively. Grain yield in soybean varied from 1508.3 kg/ha to 925 kg/ha (Table: 4.12). Highest grain

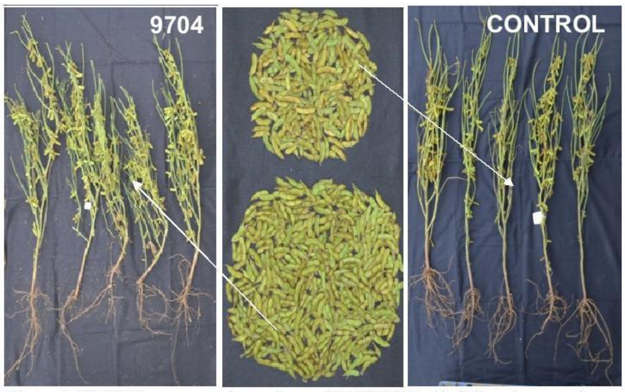


Plate: 4.18 PGP response in soybean (CG SOYA-1) following seed bacterization with flourescent *Pseudomonas* isolate 9704 under field conditions

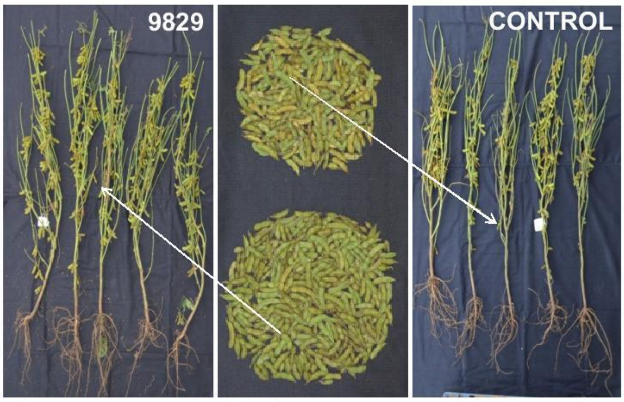


Plate: 4.19 PGP response in soybean (CG SOYA-1) following seed bacterization with flourescent *Pseudomonas* isolate 9829 under field conditions

plate: 4.18 & 4.19 PGP response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolate 9704 9829 under field conditions

Table: 4.11 Plant growth-promoting response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolates (pot experiment).

Isolate	**Shoot (cm)**	**Root (cm)**	**Branches**	**Number of filled pods**	**%Filled pods**
BS-1	$62.00^{BC}\pm1.52$	$40.33^{BCD}\pm0.88$	$5.00^{BCD}\pm0.57$	$51.00^{BCD}\pm8.50$	79.68
BS-2	$71.66^{A}\pm1.45$	$46.33^{BCD}\pm2.40$	$5.66^{ABC}\pm0.33$	$60.33^{B}\pm5.48$	79.73
BS-3	$60.00^{BCD}\pm4.48$	$59.00^{A}\pm5.68$	$4.00^{DE}\pm1.00$	$58.33^{BCD}\pm4.41$	84.95
BS-4	$54.00^{D}\pm1.76$	$51.5^{ABC}\pm3.21$	$5.00^{BCD}\pm0.66$	$45.66^{BCD}\pm6.69$	76.11
9704	$72.66^{A}\pm1.76$	$49.66^{ABC}\pm2.72$	$7.00^{A}\pm0.00$	$83.66^{A}\pm8.95$	82.83
9809	$63.50^{B}\pm0.86$	$52.5^{AB}\pm4.77$	$4.33^{CDE}\pm0.33$	$36.00^{CD}\pm3.46$	74.48
9829	$57.00^{BCD}\pm0.66$	$42.5^{BCD}\pm0.88$	$6.00^{AB}\pm0.66$	$52.33^{BCD}\pm2.33$	79.69
BSP-14	$63.16^{B}\pm0.60$	$46.5^{BCD}\pm1.75$	$5.66^{ABC}\pm0.33$	$54.00^{BCD}\pm4.72$	61.59
BSP-19	$55.00^{CD}\pm4.04$	$39.6^{CD}\pm4.84$	$5.33^{BCD}\pm0.33$	$49.33^{BCD}\pm4.97$	73.63
BSP-23	$62.33^{B}\pm0.88$	$51.6^{ABC}\pm7.86$	$4.66^{BCDE}\pm0.66$	$56.66^{BC}\pm7.21$	82.52
CONTROL	$61.00^{BCD}\pm3.77$	$36.3^{D}\pm2.96$	$3.33^{E}\pm0.33$	$33.33^{D}\pm7.83$	62.51
C.D	7.102	11.85	1.58	18.34	
SE(M)	2.406	4.014	0.54	6.21	
SE(D)	3.402	5.677	0.76	8.78	
C. V	6.737	14.83	18.6	20.39	

All the treatment values are average of three replications
All the treatments found significant at a 5 % probability level
Means followed by a common letter are not significantly different at the 5 % level
Date of sowing: 16th July 2019
Date of observation: 19th October 2019

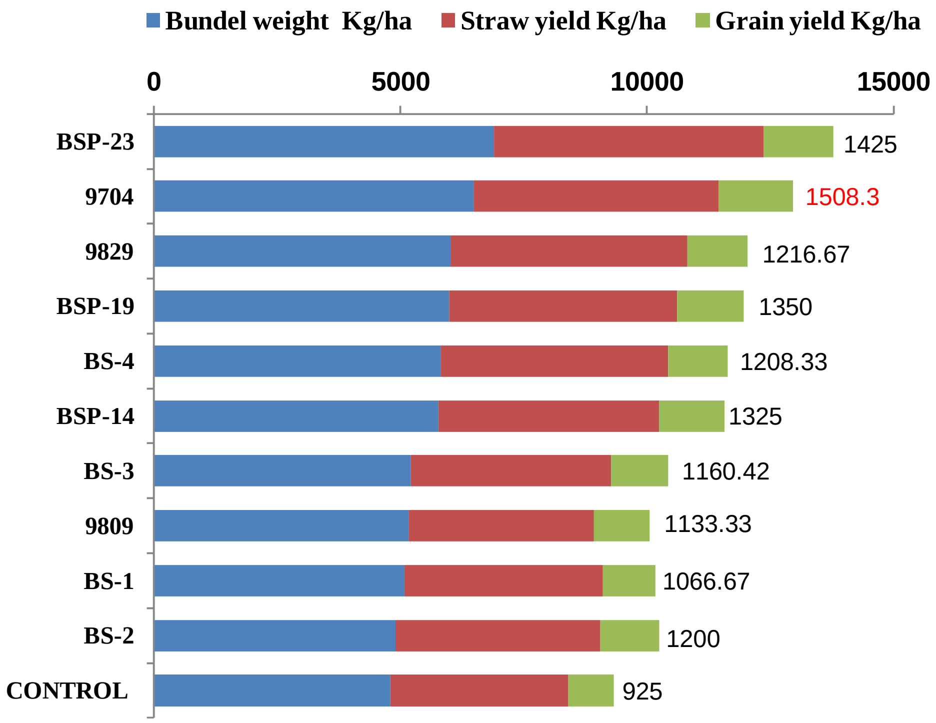
Table: 4.12 Plant growth-promoting response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolates (field experiment).

Isolates	**Shoot(cm)**	**Branches**	**Filled Pods**	**BW(Kg/ha)**	**SY(Kg/ha)**	**GW(Kg/ha)**
BS-1	$87.4^{A}\pm1.98$	$8^{AB}\pm0.31$	$89.6^{ABC}\pm4.58$	5083.33	4016.67	1066.67 (13.28%)
BS-2	$85.2^{ABC}\pm3.29$	$6.8^{BC}\pm0.37$	$76.6^{BCDE}\pm5.16$	4891.65	4158.33	1200.00 (22.92%)
BS-3	$86.2^{A}\pm1.98$	$5.8^{CD}\pm0.66$	$88.2^{ABCD}\pm7.04$	5208.33	4066.67	1160.42 (20.29%)
BS-4	$86^{AB}\pm2.78$	$9^{A}\pm0.83$	$76.2^{CDE}\pm7.13$	5816.67	4608.33	1208.33 (23.45%)
BSP-14	$86.8^{A}\pm2.88$	$5^{D}\pm0.44$	$74.4^{DE}\pm4.05$	5766.67	4483.33	1325.00 (30.19%)
BSP-19	$78.8^{C}\pm0.58$	$7^{BC}\pm0.89$	$90.6^{ABC}\pm6.25$	5983.33	4633.33	1350.00 (31.48%)
BSP-23	$83^{ABC}\pm3.23$	$8.4^{BC}\pm0.24$	$91.2^{AB}\pm2.78$	6891.67	5466.67	1425.00 (35.09%)
9829	$86.8^{A}\pm3.72$	$6.8^{BC}\pm0.58$	$70^{E}\pm5.44$	6016.67	4800.00	1216.67 (23.97%)
9809	$78.8^{C}\pm0.58$	$7.2^{AB}\pm0.66$	$68.4^{E}\pm3.98$	5166.67	3758.33	1133.33 (18.38%)
9704	$88^{A}\pm3.23$	$8^{AB}\pm0.94$	$93.4^{A}\pm2.15$	6483.33	4975.00	1508.33 (38.67%)
CONTROL	$79.2^{BC}\pm2.19$	$4.8^{D}\pm0.37$	$48.8^{F}\pm4.11$	4800.00	3600.00	925.00
C.D.	6.914	1.794	6.54	0.635	0.223	0.139
SE(M)	2.41	0.625	5.086	0.214	0.075	0.047
SE(D)	3.408	0.884	7.192	0.302	0.106	0.066
C.V.	6.4	12.02	14.329	5.462	2.455	5.489

Values of all treatments are average of five replicates
BW (Bundle weight), SY (Straw yield), and GY (Grain yield)
Variety: CG Soya-1, Plot size: 4x4m^{2},
Date of sowing: 09 /07/2019, Date of observation: 29/9/2019

Values in parenthesis are percentage increase over control
All treatments are found significant at a 5% probability level.
Seed rate: 200gm/plot, design: RBD
Means followed by a common letter are not significantly different at 5 % probability level

Figure: 4.5 Effect of fluorescent *Pseudomonas* isolates on yield attributing characters of soybean (CG SOYA-1) following seed treatment



Values of all treatments are an average of three replicates
All treatments are found significant at a 5% probability level

yield was recorded in soybean plants treated with *Pseudomonas* isolate 9704 (1508.3 kg/ha) (Plate: 4.18) which led to 38.67 % increase in grain yield followed by BSP-23 (35.09%), BSP-19 (31.48%), BSP-14 (30.19%), 9829 (23.97%), BS-4 (23.45%), BS-2 (22.92%), BS-3 (20.29%), 9809 (18.38) and BS-1(13.28%) over control or untreated soybean plants.

4.6 Plant growth-promoting response in chickpea (INDIRA CHANA-1) following seed bacterization with fluorescent *Pseudomonas* isolates

4.6.1 Pot experiment

Pot experiments were carried out at rainout shelter, College of Agriculture, I. G. K. V, Raipur during *Rabi-2019* to study the effect of *Pseudomonas* isolate on growth and yield of chickpea. The plant growth-promoting ability of ten *Pseudomonas* isolates was evaluated in soybean cultivar (INDIRA CHANA-1) following seed treatment. Ten seeds of each treatment were sowed in each pot and three replications were maintained for each treatment. Observations were recorded after 90 days of sowing (Table: 4.13). Most of the studied traits such as shoots length, root length, number of branches, and root volume were significantly different at the 5 % probability level. Pot experiment revealed that shoot length does not differ much by seed inoculation with *Pseudomonas* isolates and varied from 46.33±5.36 cm to 33.33±0.88 cm. The highest shoot length (46.33±5.36 cm), was recorded in chickpea plants treated with *Pseudomonas* isolate BSP-14. *Pseudomonas* isolates BS-4 (57.33±5.92 cm) recorded the highest root length, which led to a 16.86 % increase over control. A distinct difference was seen in the root volume of chickpea plants treated with *Pseudomonas* isolates (Plate: 4.20). The root volume of all the *Pseudomonas* treated chickpea plants varied from 246.96 cm^3 to 94.99 cm^3. Highest root volume was recorded in chickpea plant treated with *Pseudomonas* isolate BS-2 (246.96 cm^3), which led to 61.53 % increase over control followed by BS-4 (58.3%), 9704 (58.3%), BSP-14 (58.3%), 9809 (54.5%), BSP-23 (55%), 9829 (28.5%), BS-1(9%), BS-3 (9%) increase over control. Whereas, isolate BSP-19 recorded no difference in root volume in comparison to control.

4.6.2 Field experiment

Field experiments were carried out at research cum instructional farm, College of Agriculture, I. G. K. V, Raipur during *Rabi-2019* to study the effect of *Pseudomonas* isolate on growth and yield of chickpea (Plate: 4.21). Based on the results of variance analysis of chickpea field data traits like mortality %, shoot length, the number of branches, filled pods, bundle weight, strawweight, and grain yield were significantly different at the 5 % probability level (Table: 4.14). The morality of chickpea plants was recorded in field plots 27 days after sowing. The highest mortality was recorded in *Pseudomonas* untreated plots or control plots and the lowest mortality was recorded in chickpea plot treated with *Pseudomonas* isolate 9704 (2.44%) in comparison to control plots which recorded 4.27 % of mortal chickpea plants (Figure: 4.6). In a field experiment, shoot length, number of pods, bundle weight, strawweight, and grain yield parameters in chickpea are influenced by ten *Pseudomonas* isolate treatments presented in Table 4.14. A significant increase in shoot length was observed in all treatments over control. The maximum shoot length was observed in chickpea plants treated with

Pseudomonas isolate 9809 (71.34±1.69 cm), which led to 12.89% increase in shoot length over control, followed by 9.2%, 8.6%, 8.1%, 4.8%, 4.6%, 4.4%, 3.1%, 2.5% in BSP-14, 9829, BSP-19, BS-2, BSP-23, BS-4, BS-1 and BS-3 respectively. Whereas, isolate 9704 recorded a decreasing percentage of shoot length over control. Seed treatment with *Pseudomonas* isolate had significantly affected filled pods. A significant increase in filled pods was recorded in chickpea plants treated with *Pseudomonas* isolates over untreated control. Highest number of filled pods was seen in chickpea plant treated with isolate BS-4 (54.6±2.60), which led to 28.9% increase in filled pods over control, followed by 9829 (28.4%), BSP-19 (16.3%), 9704 (14.7%), BSP-14 (14.5%), BS-3 (13.5%), 9809 (10.5%), BSP-23 (10.3%), BS-2 (9.9%) and BS-1 (6.9%) increase over control.

Bundle weight, straw yield, and grain yield in chickpea plants treated with *Pseudomonas* isolates were markedly increased in comparison to untreated or control (Figure: 4.7). The highest bundle weight (4416.66 kg/ha) was recorded in chickpea plants treated with *Pseudomonas* isolate BS-4 led to a 26.41 % increase over control. The highest straw yield (3352.16 kg/ha) was recorded in chickpea plants treated with *Pseudomonas* isolate BS-2 led to a 24.38 % increase over control. Grain yield in chickpea varied from 1153.33kg/ha to 715.5 kg/ha (Table: 4.14). Highest grain yield was recorded in chickpea plants treated with *Pseudomonas* isolate BS-4 (1153.33kg/ha) which led to 37.9 % increase in grain yield followed by 9704 (35.7%), BS-1 (32.6%), BS-3 (32.6%), 9829 (28.4%), BS-2 (27%), BSP-14 (21%), BSP-23 (15.8%), BSP-19 (15.4%) and 9809 (7.4%) over control or untreated chickpea plants. According to the result of the variance analysis harvest index was affected by all *Pseudomonas* treated chickpea plants. The maximum harvest index was recorded in chickpea plants treated with *Pseudomonas* isolate 9704 (27.58%) and this response was probably due to higher grain yield and biomass. However, the lowest harvest index was recorded in untreated chickpea plants (22.01%).

Plate: 4.20 PGP response in chickpea (INDIRA CHANA-1) following seed bacterization with flourescent *Pseudomonas* isolates under pot conditions

Plate: 4.20 PGP response in chickpea (INDIRA CHANA-1) following seed bacterization with fluorescent Pseudomonas isolates under pot conditions

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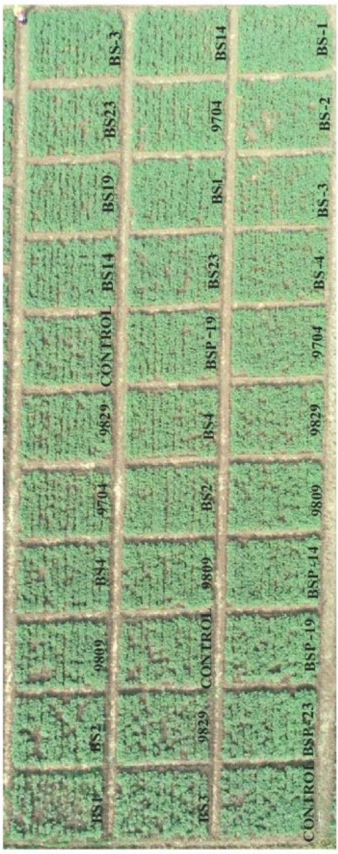


Plate: 4.21 Efficacy of different fluorescent *Pseudomonas* isolates on plant growth promoting effects of chickpea (Indra chana-1)

Plate: 4.21 Efficacy of different fluorescent *Pseudomonas* isolates on plant growth-promoting effects of chickpea (Indra chana-1)

Table: 4.13 Plant growth-promoting response in chickpea (INDIRA CHANA-1) following seed bacterization with fluorescent *Pseudomonas* isolates (pot experiment).

Isolates	**Root length (cm)**	**Shoot length (cm)**	**Branches**	**Root volume (cm^3)**
BS-1	44±3.78^{BC}	34.667±0.66^{B}	4.667±0.33^{CD}	104.48^{CD}
BS-2	49.66±5.23^{AB}	35±1.73^{B}	5.333±0.66^{ABCD}	246.96^{A}
BS-3	40.66±4.97^{BCD}	38±2.64^{B}	5±0.57^{BCD}	104.48^{CD}
BS-4	57.33±5.92^{A}	35.333±1.20^{B}	4.33±0.33^{DE}	227.96^{AB}
9704	47.66±4.33^{AB}	34.667±0.33^{B}	5.33±0.33^{ABCD}	227.96^{AB}
9829	33.33±1.66^{D}	37.667±1.45^{B}	6±0.57^{AB}	132.98^{C}
9809	44.66±1.76^{B}	39±1.52^{B}	5.66±0.33^{ABC}	208.97^{B}
BSP-14	48.66±0.88^{AB}	46.333±5.36^{A}	5±0.57^{BCD}	227.96^{AB}
BSP-19	33.66±2.96^{CD}	35.667±1.85^{B}	4.33±0.33^{DE}	94.99^{E}
BSP-23	48.66±0.88^{AB}	36.667±2.18^{B}	6.33±0.33^{A}	208.97^{B}
CONTROL	47.66±1.20^{AB}	33.333±0.88^{B}	3.33±0.33^{E}	94.99^{E}
CD	10.393	6.5	1.32	
CV	13.611	10.41	15.4	

All the treatment values are average of three replications,
All the treatments found significant at a 5 % probability level
Variety: Indira chana-1
Site: rainout shelter IGKV Raipur
Date of sowing: 20/11/2019
Date of observation: 20/02/2020

Table: 4.14 Plant growth-promoting response in chickpea (INDIRA CHANA-1) following seed bacterization with fluorescent *Pseudomonas* isolates (field experiment).

Isolate	**Mortality (%)**	**Shoot length (cm)**	**Branches**	**Pods/plant**	**BW (kgha^{-1})**	**SY (kgha^{-1})**	**GY (kgha^{-1})**	**HI (%)**
BS-1	3.69	64.185±1.49^{D}	4.7±0.26^{DEF}	41.5±2.74^{BCD}	4000.00^{BCD}	2937.00^{BC}	1063.00^{AB}	26.58
BS-2	3.78	65.287±1.37^{CD}	4.8±0.24^{CDEF}	43.1±1.14^{BC}	4333.00^{AB}	3352.16^{A}	981.16^{BC}	22.64
BS-3	4.26	63.74±1.25^{DE}	4.7±0.26^{DEF}	44.9±2.03^{B}	4166.66^{ABC}	3105.83^{AB}	1061.83^{AB}	25.48
BS-4	4.1	65.033±2.48^{CDE}	5.7±0.21^{AB}	54.6±2.60^{A}	4416.66^{A}	3263.33^{AB}	1153.33^{A}	26.11
9704	2.44	61.993±0.96^{DEF}	5.5±0.26^{ABC}	45.5±2.22^{B}	4000.00^{BCD}	2885.83^{BCD}	1114.16^{A}	27.58
9829	3.83	67.867±1.33^{BC}	5.7±0.30^{AB}	54.2±1.19^{A}	4000.00^{BCD}	2999.66^{ABC}	1000.33^{BC}	25.00
9809	3.26	71.347±1.69^{A}	5.2±0.24^{BCDE}	43.4±2.45^{BC}	3666.66$_{DE}$	3160.16^{BCD}	773.10^{EF}	21.08
BSP-14	3.17	68.453±1.18^{B}	5.3±0.39^{ABCD}	45.4±1.60^{B}	3583.33^{EF}	2677.16^{CD}	906.00^{CD}	25.28
BSP-19	3.53	67.653±1.66^{BC}	6.0±0.25^{A}	46.4±2.63^{B}	3833.33^{CDE}	3154.00^{ABC}	846.00^{DE}	22.07
BSP-23	3.12	65.207±2.57^{CD}	4.5±0.22^{EF}	43.3±2.23^{BC}	3500.00^{EF}	2650.00^{CD}	850.00^{DE}	24.28
CONTROL	4.27	62.14±1.52^{F}	4.3±0.15^{F}	38.8±1.34^{C}	3250.00^{F}	2534.66^{D}	715.50^{G}	22.01
C.D.		4.694	0.741	6.082	0.79	0.784	0.209	
SE(m)		1.677	0.263	2.161	0.2	0.264	0.07	
SE(d)		2.371	0.372	3.056	0.3	0.373	0.1	
C.V.		9.882	16.24	15.003	5.97	7.784	6.417	

Values of Plant height, branches, and pods are an average of 10 replicates

Values of BW (Bundle weight), SY (Straw yield), and GY (Grain yield) are average of three replicates

All treatments are found significant at a 5% probability level.

Variety: Indra chana-1; Site of experiment field: Bharri farm; Plot size: 4x5m^{2}; Seed rate: 200grams/plot

Date of sowing: 30 /11/2019; Date of observation: 25/3/2020; Design: RBD

Figure: 4.6 Effect of seed bacterization with *Pseudomonas* isolates on mortality the rate in chickpea (INDRA CHANA-1).

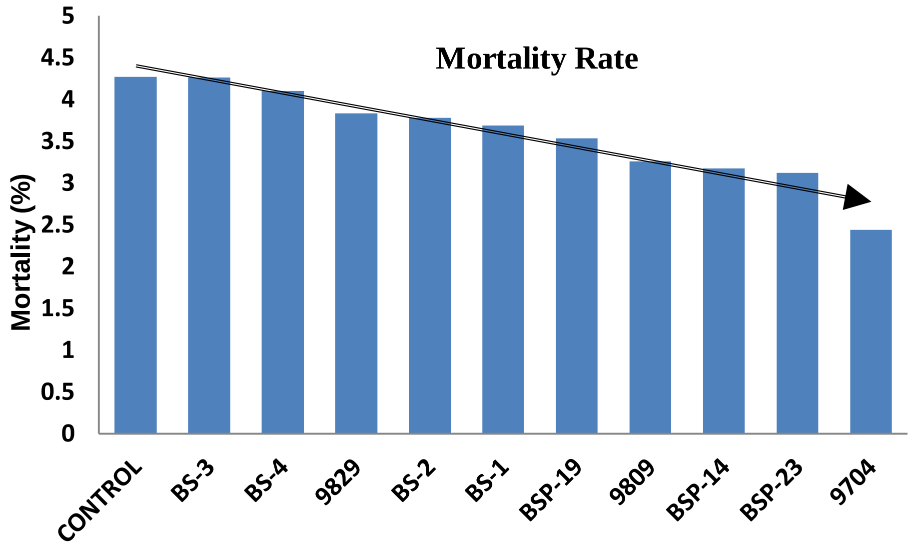
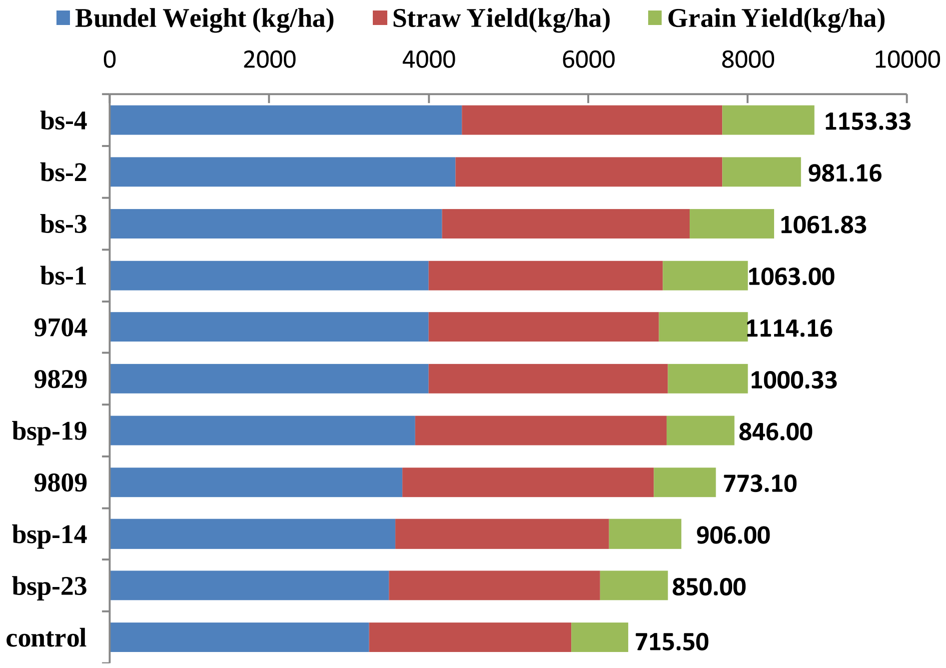


Figure: 4.7 Affect of fluorescent *Pseudomonas* isolates on yield attributing characters in chickpea (INDRA CHANA-1) following seed treatment



Values of all treatments are an average of three replicates
All treatments are found significant at a 5% probability level

4.7 Plant growth-promoting response in wheat (AMBER WHEAT) following seed bacterization with fluorescent *Pseudomonas* isolates.

4.7.1 Field experiment

Field experiments were carried out at research cum instructional farm, College of Agriculture, I. G. K. V, Raipur during *Rabi-2019* to study the effect of *Pseudomonas* isolate on the growth and yield of wheat. Based on the results of variance analysis of wheat field data traits like shoot length, shoot fresh weight, shoot dry weight, number of productive tillers, grain number per spike, 1000 seed weight, bundle weight, strawweight, and grain yield and harvest index were significantly different at 5 % probability level (Plate: 4.22). Infield experiment, all the parameters attributing growth and yield in amber wheat were influenced by two *Pseudomonas* isolate treatments presented in Table: 4.15. A significant increase in shoot length was observed in both the treatments over control. The maximum shoot length was observed in wheat plants treated with *Pseudomonas* isolate 9829 (82.48±1.22 cm), which led to a 7.14% increase in shoot length over control, followed by *Pseudomonas* isolate 9704 which led to a 4.3%, increase in shoot length over control (Plate: 4.22). Seed treatment with *Pseudomonas* isolate had significantly affected shoot fresh and dry weights of wheat plants. A significant increase in shoot fresh and dry weight was recorded in wheat plants treated with *Pseudomonas* isolates over untreated control (Figure: 4.8). Highest shoot fresh weight (96.7±1.5gm) and shoot dry weight (61.4±1.29gm) in wheat plants treated with isolate 9829, which led to 29.8% and 18.4%, increase over control respectively. Seed treatment with *Pseudomonas* isolate had also led to an increase in grain number per spike in each tiller of wheat plants in comparison to control or untreated. The highest number of grains per spike had recorded in wheat plants treated with *Pseudomonas* isolate 9829 (58.7±0.94), which led to a 9.7% increase over control followed by *Pseudomonas* isolate 9704 (56.4±1.83), which led to a 6% increase over control. Bundle weight, straw yield, and grain yield in wheat plants treated with *Pseudomonas* isolates were markedly increased in comparison to untreated or control. The highest bundle weight (7536.5kg/ha) and straw yield (4099.81kg/ha) were recorded in wheat plants treated with *Pseudomonas* isolate 9829 led to 18.01% and 20.1% increase over control respectively.

Plate: 4.22 PGP response in wheat (CG Amber wheat) following seed bacterization with flourescent *Pseudomonas* isolates under field conditions

Plate: 4.22 PGP responses in wheat (CG Amber wheat) following seed bacterization with fluorescent Pseudomonas isolates under field conditions

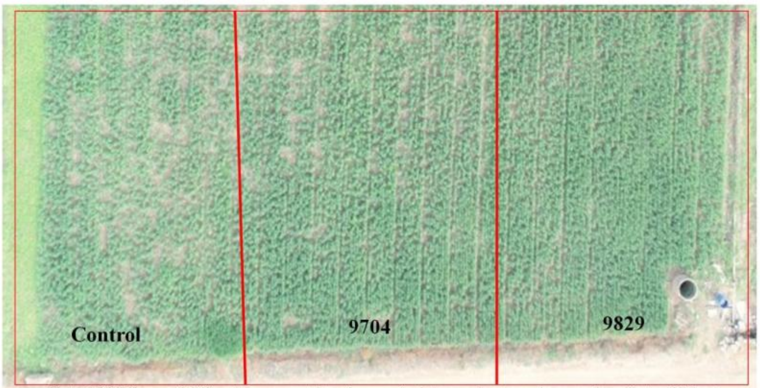


Plate: 4.23 Efficacy of different fluorescent *Pseudomonas* isolates on plant growth promoting effects of Wheat

Plate: 4.23 Efficacy of different fluorescent Pseudomonas isolates on plant growth-promoting effects of Wheat

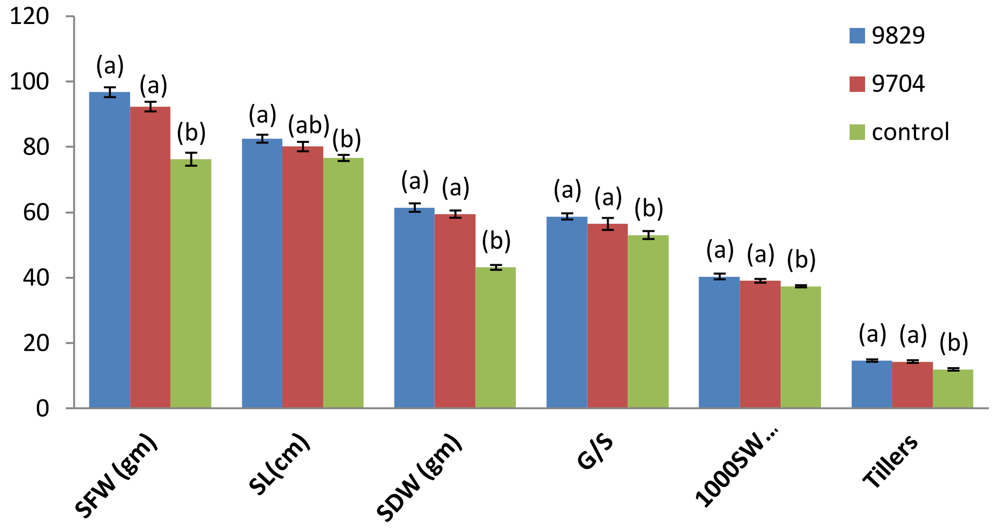
Table: 4.15 Plant growth-promoting response in wheat (AMBER WHEAT) following seed bacterization with fluorescent *Pseudomonas* isolates (field experiment).

Isolate	**Shoot (cm)**	**Shoot fresh weight (gm)**	**Shoot dry weight(gm)**	**No. of productive tiller**	**Grain no/spike**	**1000 seed weight**	**BW Kg/ha**	**SY Kg/ha**	**GY Kg/ha**	**HI %**
9829	82.48±1.22^{A}	96.7±1.51^{A}	61.4±1.29^{A}	14.6±0.37^{A}	58.7±0.94^{A}	40.33±0.88^{A}	7536.51^{A}	4099.81^{A}	3446.44^{A}	45.60
9704	80.07±1.44^{AB}	92.3±1.47^{A}	59.4±1.11^{A}	14.3±0.41^{A}	56.4±1.83^{A}	39.00±0.57^{A}	6960.56^{B}	3703.78^{AB}	3265.88^{A}	47.33
control	76.59±0.94^{B}	76.2±1.99^{B}	43.1±0.74^{B}	11.9±0.39^{B}	53±1.22^{B}	37.33±0.33^{B}	6178.61^{C}	3275.04^{C}	2909.78^{B}	46.99
CV	5.4	6.4	6.8	12.1	5.85	2.26	7.8	8.9	6.2	
CD	4.05	5.3	3.5	1.54	3.08	2.05	2.39	1.45	0.89	

All values are the average of 10 replicates
All treatments are found significant at 5% probability level
BY: Biological yield, SY: Straw yield, GY: Grain yield, HI: Harvest index
Date of observation: 18/3/2020

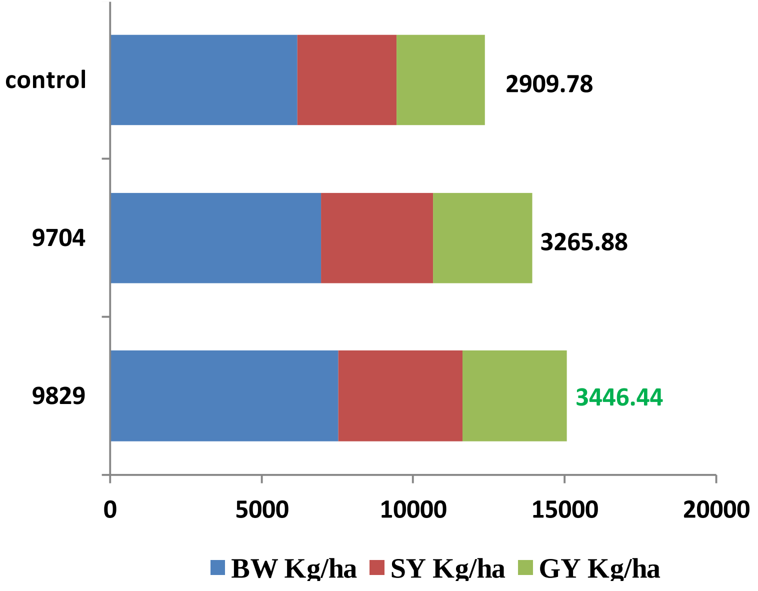
Variety: CG Amber wheat
Site of experiment field: NSP (National Seed Project)
Plot size: 13X20.5m^{2}, Date of sowing: 29/11/2019
Design: RBD

Figure: 4.8 Plant growth-promoting response in wheat (AMBER WHEAT) following seed bacterization with fluorescent *Pseudomonas* isolates.



All values are the average of 10 replicates
All treatments are found significant at a 5% probability level
Means followed by a common letter are not significantly different at a 5 % level

Figure: 4.9 Effect of fluorescent *Pseudomonas* isolates on yield attributing characters in wheat (AMBER WHEAT) following seed treatment



BY: Biological yield, SY: Straw yield, GY: Grain yield
Values of all treatments are an average of seven replicates
All treatments are found significant at a 5% probability level

Grain yield in wheat varied from 3446.44 kg/ha to 2909.78kg/ha. The highest grain yield was recorded in wheat plant treated with *Pseudomonas* isolate 9829 (3446.44kg/ha) which led to a 15.5% increase in grain yield followed by 9704 (10.9%) (Figure: 4.9). According to the result of the variance analysis harvest index was affected by all *Pseudomonas* treated wheat plants. The maximum harvest index was recorded in wheat plants treated with *Pseudomonas* isolate 9704 (47.33%) and this response Grain yield in wheat varied from 3446.44 kg/ha to 2909.78kg/ha was probably due to higher grain yield and biomass.

In the present investigation, ten *Pseudomonas* isolate were evaluated for their growth and yield attributing characters in soybean, chickpea, and wheat. All the isolates produced diffusible greenish-yellow pigments, which fluorescence under UV light on King's B medium. These fluorescent *Pseudomonas* isolates were screened for siderophore production and BSP-19 (79.71%) produced the highest percent siderophore unit (Table: 4.8). The presence of siderophore in fluorescent *Pseudomonas* has been reported by several authors (Bakker and Schippers 1987; Bezbaruah 1994). The highest production of IAA by *Pseudomonas* isolates BSP-23 (Table: 4.7) Oberhänsli et al (1991) reported the production of IAA by *P. fluorescens* isolated from tobacco rhizosphere. All the *Pseudomonas* isolates caused phosphate solubilization, 9704 being the best (Table: 4.6). Kloepper et al (1988) reported solubilization of phosphorous as the most readily available form for plant growth. *Pseudomonas* isolate 9704 (60% inhibition) showed *in-vitro* antagonism against *M. phaseolina* by performing confrontation assay (Kotasthane et al. 2017) and modified Petri plate assay.

Fluorescent *Pseudomonas* has been shown to produce an arsenal of metabolites that maintain plant health and increase plant growth and root development. However, most of the findings are limited to pot experiments (Bagnasco et al. 1998; O'sullivan and O'Gara 1992; Dowling and O'Gara 1994). Ten *Pseudomonas* isolates tested in field trails produced an arsenal of metabolites and induced plant growth and yield. Infield experiments seed bacterization with *Pseudomonas* isolates has enhanced the growth of soybean, chickpea, and wheat (Kumar and Dube 1992; Saber et al. 2012).

Seed bacterization with *Pseudomonas* isolate had enhanced seed germination in soybean (Table: 4.8). The results suggest that *Pseudomonas* isolate 9704 is a plant growth-promoting rhizobacteria that have the potential for use in seed bacterization for higher germination and growth of soybean seedling. These findings may be due to the increased synthesis of hormones by *Pseudomonas* isolate 9704. Gibberellins trigger the activity of specific enzymes such as amylase, which increases the availability of starch that promoted early germination (Gholami et al. 2009). (Malleswari and Bagyanarayana (2013) studied that seed inoculation initiates physiological processes of germination and helps in the proliferation of bioagent in the spermosphere. Therefore using *Pseudomonas* isolates as seed inoculants useful in enhancing germination in soybean.

Shoot and root characters were significantly improved in soybean, chickpea, and wheat cultivars treated with *Pseudomonas* isolates. *Pseudomonas* isolated from *Brassica* specific (BS-2) rhizosphere showed enhanced root growth in soybean and chickpea. Kalbe et al (1996) studied that *Pseudomonas* isolated from *B. napus* rhizosphere was shown to possess antifungal properties and to secrete the plant growth hormone indole acetic acid that can directly promote root growth. Low concentration of IAA can stimulate primary root elongation, whereas, high IAA levels stimulate the formation of lateral roots, decrease primary root length and increase root hair formation (Patten and Glick. 2012; Dobbelaere et al. 1999).

Field experimental results of seed treatment with *Pseudomonas* isolates in chickpea, soybean and wheat revealed a significant increase in yield and total biomass. Seed bacterization with *Pseudomonas* isolates 9704 had increased grain yield in soybean (CG-SOYA1) by 38.6 % over control or untreated soybean plants. Seed bacterization with *Pseudomonas* isolates BS-4 had increased grain yield in chickpea (INDIRA CHANA-1) by 37.9 % over control or untreated chickpea plants. Seed bacterization with *Pseudomonas* isolates 9829 had increased grain yield in wheat (Amber wheat) by 15.57 % over control or untreated wheat plants. Similar results of a significant increase in yield by seed inoculation of bioagent were found by (Elekhtyar 2015; Gholami et al. 2009; Saber et al. 2012; Shweta et al. 2008).

4.8 Effect of seed bacterization with *Pseudomonas* isolates in chickpea and wheat leaves contents of superoxide dismutase (SOD), peroxidase (POX), phenylamine ammonia-lyase (PAL), lipid peroxidase (LPO), polyphenol oxidase (PPO), and total phenolic content (TPC).

Many authors showed that microorganisms induce antioxidant in response to various stresses (Singh et al. 2014; Seo et al. 2012; Nawrocka et al. 2011; Mandal et al. 2008; Benhamou et al. 2002). Oxidative stress is induced in plants by a wide range of abiotic (light/dark, temperature, nutrients, water availability, a toxic compound such as heavy metal) and biotic interactions (beneficial and pathogenic microbes, fungi, insects, and other factors) (Suzuki et al. 2014). Such induced stress leads to an increase in the production of ROS (reactive oxygen species) that leads to an imbalance in production and scavenging of ROS. Increased ROS easily reacts with various biomolecules, causing irreversible damage and leading to necrosis and plant death (Pitzschke et al. 2006). To tackle excessive ROS production, plants produce different enzymatic (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), phenylalanine lyases (PAL), and non-enzymatic antioxidant (total phenolic content (TPC), polyphenol oxidase (PPO)) that scavenges oxygen species. Previously it was demonstrated that non-pathogenic rhizobacteria such as *Pseudomonas* spp. protect the plant from diverse stresses by inducing antioxidant enzymes that scavenge ROS (reactive oxygen species) (Sarma et al. 2002; Singh et al. 2003).

In the present investigation, we had evaluated induction in antioxidant enzymes upon seed bacterization with *Pseudomonas* isolates in chickpea and wheat plants over control. Mature and fresh flag leaves of chickpea and wheat plants were used to estimate the quantity of antioxidant enzyme by spectrophotometric analysis.

4.8.1 Superoxide dismutase (SOD)

SOD is an endogenous, detoxifying, and powerful antioxidant enzyme in the cell. SOD acts as the first line of defense system against ROS (reactive oxygen species). Superoxide dismutase catalyzes highly reactive form superoxide anion ($^{-}O_2$) to less toxic hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Alscher et al. 2002; Ighodaro and Akinloye 2018). Spectrophotometric analysis of ROS scavenging

Table: 4.16 Effect after seed bacterization with *Pseudomonas* isolates in chickpea and wheat leaves contents of superoxide dismutase (SOD), Lipid peroxidase (LPO), phenylalanine ammonia-lyase (PAL), peroxidase (POx), polyphenol oxidase (PPO), and total phenolic content (TPC).

Treatments	**SOD**	**POx**	**PAL**	**LPO**	**PPO**	**TPC**
(a)Chickpea	**Units/g**	**ΔOD min^{-1}g^{-1}FW**	**mmole/L/g**	**nmole/L/g**	**ΔOD min^{-1}g^{-1}FW**	**µg GAE/g**
BS-1	959.49^{BC}±156.81	89.5141	3.009^{ABC}±0.002	11500DE±900	0.00063	1265.08^{B}±11.92
BS-2	498.196^{D}±186.44	87.0393	2.991^{BCD}±0.001	17650^{BC}±3150	0.00317	1316.33^{AB}±28.41
BS-3	1130.37^{B}±147.50	52.0648	3.024^{A}±0.002	24500^{A}±2700	0.00285	1080.79^{CD}±1.375
BS-4	263.01^{D}±156.47	61.5906	3.013^{ABC}±0.016	20500^{AB}±300	0.00419	1053.33^{D}±11.67
9704	967.01^{BC}±165.54	68.3613	3.019^{AB}±0.009	15800^{CD}±0.00	0.00293	1375.75^{A}±10
9829	644.52^{CD}±341.24	62.9914	3.017^{AB}±0.006	13650^{CDE}±550	0.00269	1271.5^{B}±7.67
9809	646.58^{CD}±34.58	73.0775	2.99^{BCD}±0.004	15300^{CD}±400	0.00387	1154.96^{C}±25.46
BSP-14	1047.50^{B}±183.68	76.5329	3.03^{A}±0.011	9700^{E}±300	0.00215	696.96^{F}±16.04
BSP-19	1546.28^{A}±175.18	56.1740	3.004^{ABC}±0.003	17950^{BC}±650	0.00505	1273.66^{B}±45.83
BSP-23	290.64^{D}±166.55	53.7925	2.973^{D}±0.014	22600^{A}±1500	0.00366	1048.75^{DE}±51.17
CC	962.60^{BC}±52.17	73.5912	2.986^{CD}±0.015	11950DE±50	0.00288	958.25^{E}±44.17
(b)Wheat						
9704-W	1526.73^{B}±76.94	79.8016	3.072^{A}±0.049	29600^{A}±3800	NA	1297.92^{A}±38.75
9829-W	1419.48^{A}±105.29	101.0945	3.072^{AB}±0.043	24150^{B}±3050	NA	1242.96^{A}±58.79
CW	2108.31^{B}±33.81	97.8725	2.991^{B}±0.106	21450^{C}±2050	NA	697.37^{B}±30.04

GAE= Gallic acid equivalent; ΔOD min^{-1}g^{-1}FW= change in OD min^{-1}g^{-1}Fresh weight; values of LPO, PAL, SOD & TPC are average of three replications; values after ± represent standard error (SE); CC= Chickpea control; CW= wheat control.

the activity of SOD was carried out in chickpea and wheat samples derived from seed bacterization with *Pseudomonas* isolates (Table: 4.16a and b). NBT (nitroblue tetrazolium) reduction is used as an indicator of O_2^- (superoxide anions) production. SOD competes with NBT for O_2^-. The percent inhibition of NBT reduction is a measure of the amount of SOD present (units/1g). Induction of SOD activity was noticed in chickpea samples derived from seed bacterization with *Pseudomonas* isolates.

Chickpea plants treated with *Pseudomonas* isolates BSP-19 (1546.28±175.18 Unit/g) and BS-3 (1130.37^B±147.50 Unit/g) showed maximum SOD activity leading to 37.7% and 14.8% increase over control respectively. All the isolates except BSP-19 and BS-3 in chickpea showed lower SOD production than the control (Figure: 4.10). In wheat samples derived from seed bacterization with *Pseudomonas* isolates 9829 and 9704 SOD production was less as compared control (Figure: 4.11) indicating that *Pseudomonas* isolates were ineffective in inducing SOD production in wheat. Similar results of an increase in SOD activity in *Cucumis sativus L.*was recorded by Helepciuc et al (2014). Cucumber plantlets treated with beneficial bacteria strain B40 (*B. licheniformis*) and P14 (*P. aeruginosa*) recorded maximum accumulation of SOD, besides lignin production and activation of defense mechanism. Singh et al (2014) also recorded that chickpea plants treated with the triple microbial consortium (*fluorescent Pseudomonas*+ *T. harzianum* THU0816+ *Rhizobium leguminosarum* RL091) resulted in 1.76 and 3.60 folds increase in SOD activity in pathogen challenged and non challenged control plants respectively.

4.8.2 Peroxidase (POX)

Peroxidases scavenge H_2O_2 (hydrogen peroxide by two-electron oxidation-reduction processes concomitant with the oxidation of phenolics and non-phenolic substrate (Goodwin et al. 1997; Henriksen et al. 1999). Class III peroxidase plays important role in plant growth and development including ROS metabolism (Pandey et al. 2017). Thus peroxidases are involved in various physiological processes such as cell wall metabolism, lignifications, suberization, and defense against pathogen

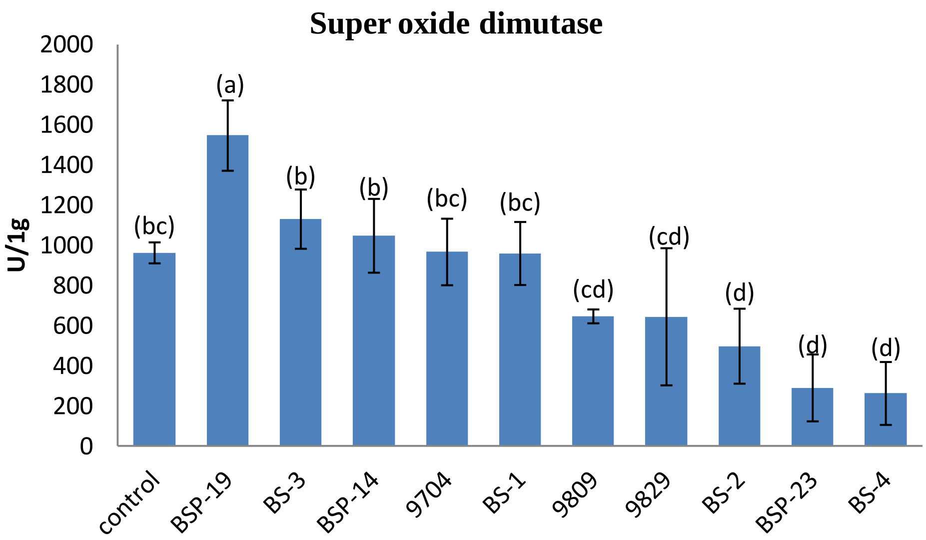


Figure: 4.10 Effect of seed bacterization of *Pseudomonas* isolates on the activity of Superoxide dismutase (SOD) in chickpea leaves

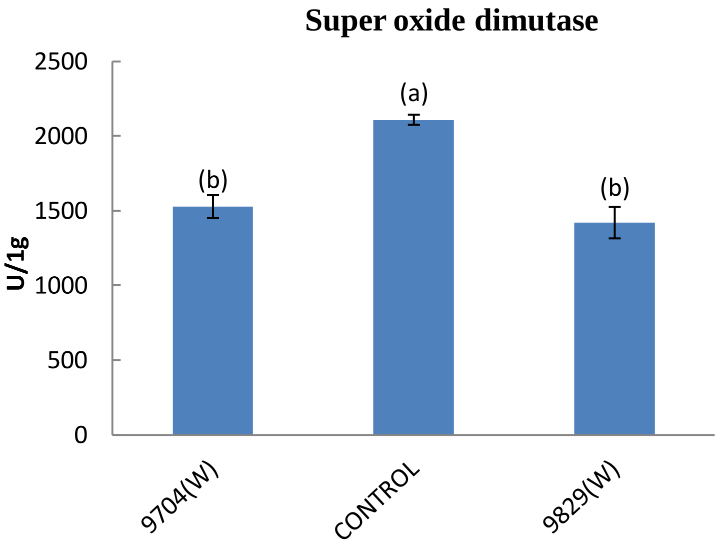


Figure: 4.11 Effect of seed bacterization of *Pseudomonas* isolates on the activity of Superoxide dismutase (SOD) in wheat leaves

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level
*Means followed by a common letter are not significantly different at 5 % level

(Bernards et al. 1999; Barceló and Pomar 2001; Passardi et al. 2004). The present investigation on peroxidase activity revealed that variation in levels of accumulation of POX ($min^{-1}g^{-1}FW$) was consistently higher in chickpea and wheat samples derived from *Pseudomonas* isolates (Table: 4.16a, 4.16b) Maximum POX activity was observed in samples derived from seed bacterization with *Pseudomonas* isolate BS-1 (89.5141 $min^{-1}g^{-1}FW$) in chickpea plants leading to a 17.78% increase in POX activity over control, followed by BS-2 (87.0393 $min^{-1}g^{-1}FW$) and BSP-14 (76.5329 $min^{-1}g^{-1}FW$). Other isolates could not induce increased POX production over control (Figure: 4.12). Similarly in wheat also maximum POX activity was observed in samples derived from seed bacterization with *Pseudomonas* isolate 9829 (101.0945 $min^{-1}g^{-1}FW$) leading to a 3.18% increase in POX activity over control (Figure: 4.13).

Similar results of an increase in POX activity upon treatment with bioagent consortium were reported by Singh et al (2014). Bioagent treatment in chickpea plants showed enhanced activity of POX but in the triple consortium (*fluorescent Pseudomonas*+ *T. harzianum* THU0816+ *Rhizobium leguminosarum* RL091) 1.40 and 2.40 folds increased activity was observed in pathogen challenged and non-challenged healthy control plants respectively. Anand et al (2007) also recorded an increase in PO activity in *P.fluorescent* treated tomato plants challenge inoculated with *A. solani* and *S. lycopersici.*

4.8.3 Phenylalanine ammonia lyases (PAL)

The ubiquitous feature of plant response to different biotic and abiotic stress is the activation of the phenylpropanoid pathway in which PAL catalyzes the first step in phenylpropanoid metabolism. Phenylpropanoid metabolism starts with the conversion of L-Phenylalanine into trans-cinnamic acid by PAL and leads to the synthesis of compounds that have a diverse function in plants, notably in defense, antimicrobial activity (isoflavonoid, phytoalexins), strengthening cell wall by the synthesis of lignin and suberin and also as signaling compounds such as salicylic acid (Massala *et al.,* 1980; Hammerschmidt, 1999). The present investigation revealed that variation in levels of accumulation of PAL was consistently higher in chickpea and wheat samples derived from seed bacterization with *Pseudomonas* isolates.

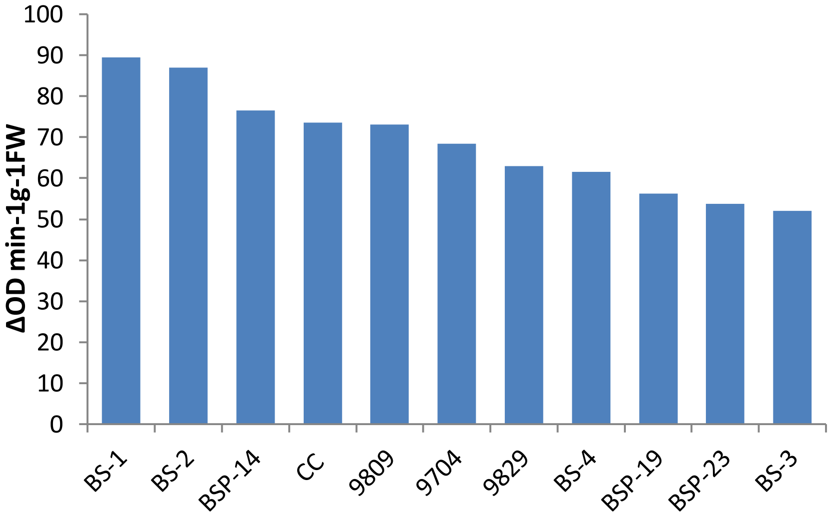


Figure: 4.12 Effect of seed bacterization of *Pseudomonas* isolates on the activity of peroxidase (POx) in chickpea leaves

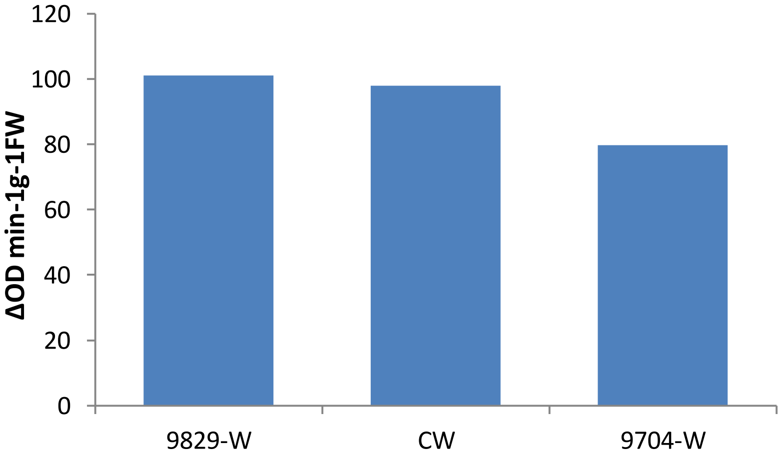


Figure: 4.13 Effect of seed bacterization of *Pseudomonas* isolates on the activity of peroxidase (POx) in wheat leaves

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level

A significant increase in PAL (mmole/L/g) activity was noticed in chickpea and wheat plants pre-treated with *Pseudomonas* isolates (Table: 4.16a, 4.16b). Seed bacterization with *Pseudomonas* isolates showed higher PAL enzyme activity.

4.8.4 Lipid Peroxidase (LPO)

Reactive oxygen species can induce lipid peroxidation and disrupt the bilayer lipid membrane arrangement and increase tissue permeability (Girotti 1985). The main targets of ROS in phospholipid membrane are the double bond between C- atoms and the ester linkage between glycerol and fatty acids. Lipid peroxides are unstable and decompose to form a complex series of a compound including reactive carbonyl compounds. The PUFA (polyunsaturated fatty acid) like linoleic and linolenic acid of the lipid membrane are hot spots for ROS damage and highly prone to hydroxyl radicals. The PUFA (polyunsaturated fatty acid) generate malondialdehyde (MDA) upon decomposition. Measurement of MDA has been used as an indicator of lipid peroxidation (Das and Roychoudhury 2014). The present investigation revealed that variation in levels of accumulation of malondialdehyde was consistently higher in chickpea and wheat samples derived from seed treatments with *Pseudomonas* isolates.

Spectrophotometric analysis of chickpea and wheat samples derived from seed bacterization with *Pseudomonas* isolates showed maximum accumulation of malondialdehyde (n mole/L/g) (Table: 16a, 16b). All the *Pseudomonas* isolates except BS-1 and BSP-14 in chickpea samples and isolates 9704 in the wheat sample showed increased accumulation of malondialdehyde in comparison to control samples. Among all the isolates of *Pseudomonas*, BS-3 (24500±2700 n mole/L/g) in chickpea (Figure: 4.16) and isolate 9704 (29600±3800 nmol/L/g) in wheat samples (Figure: 4.17) which led to51.2% and 27.53 increase over control respectively showed maximum accumulation of malondialdehyde. Similar results of the accumulation of MDA in chickpea were also found by Singh et al (2014). A decrease in MDA was detected in all plants treated with biocontrol agents throughout the experimental period compared to untreated plants challenged with the pathogen. A maximum decrease in MDA by 0.79 fold concerning challenged control plants was observed in PHU 094+THU0816 at 96hrs.

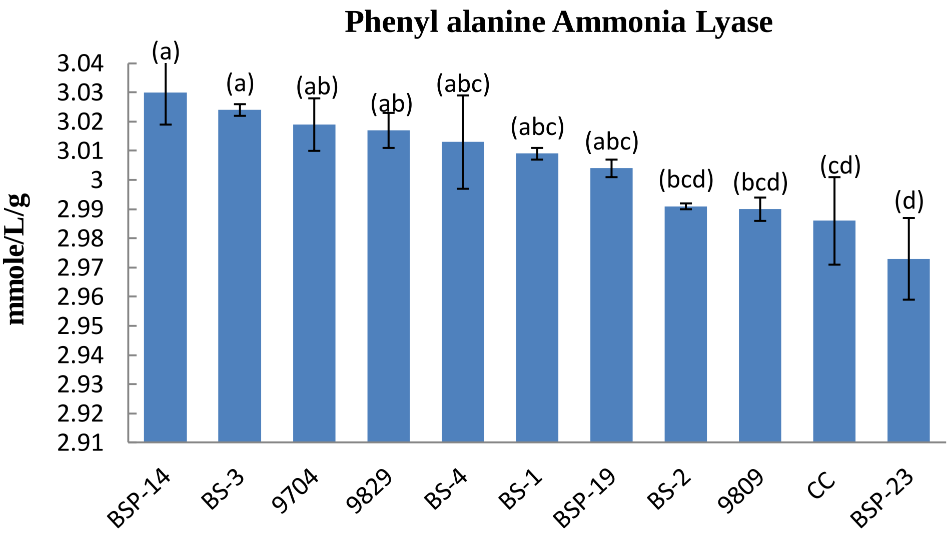


Figure: 4.14 Effect of seed bacterization of *Pseudomonas* isolates on the activity of phenylalanine ammonia-lyase (PAL) in chickpea leaves

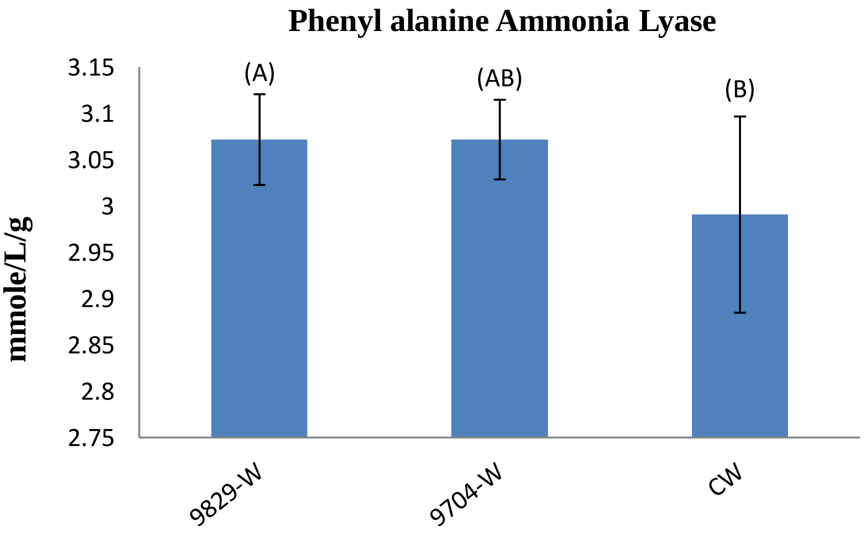


Figure: 4.15 Effect of seed bacterization of *Pseudomonas* isolates on the activity of phenylalanine ammonia-lyase (PAL) in wheat leaves

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level
*Means followed by a common letter are not significantly different at 5 % level
*Error bars indicate one standard error of the mean

4.8.5 Polyphenol oxidase (PPO)

PPOs catalyze the oxidation of monophenols and or o-diphenols to highly reactive o-quinones, which in turn interact with oxygen to form reactive oxygen species and typical brown pigment complex. Hence PPOs have an inhibitory effect on the local level of oxygen and reactive oxygen species (Yoruk and Marshall 2003; Thipyapong et al. 2004). The present investigation revealed that variation in levels of accumulation of PPOs was consistently higher in chickpea samples derived from treatment with *Pseudomonas* isolates (Table: 4.16a, 4.16b). An increase in PPO activity was observed in chickpea and wheat samples pre-treated with *Pseudomonas* isolates. Seed bacterization with *Pseudomonas* isolates BS-1, BS-3, 9829 and BSP-14 could not induce PPO production in comparison to control or untreated plants. However, samples derived from seed bacterization with *Pseudomonas* isolate BSP-19 (0.00505 $min^{-1}g^{-1}FW$) led to an increase in PPO activity of up to 42.97% in chickpea samples in comparison to control. Similar results of induction of PPOs activity was reported by Meena et al. (2000) *Pseudomonas fluorescence* induced the activity of PPOs in response to infection by *Cercospora personatum* in groundnut. Arnnok et al. (2010) also found an increase in PPO activity in hot pepper pericarp. Naqvi et al (2013) evaluated PPO activities by enzymatic assays in grains of different wheat genotypes using different PPO substrate.

4.8.6 Total phenolic content (TPC)

Phenolics are the most important groups of secondary metabolites found in plants. Plants in response to stress produce phenols accumulated in the sub-epidermal layer of plant tissues (Cle et al., 2008). Phenolics have been considered more powerful antioxidant than vitamin C, E, carotenoids and have the property of scavenging radical species such as ROS (reactive oxygen species) as polyphenols possess phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to free radical (Dai and Mumper,2010; Rice-evans *et al.*,1995). Phenolic compounds (POH) act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by the rapid donation of a hydrogen atom to radicals. Lignin constitutes a phenolic compound that enhances

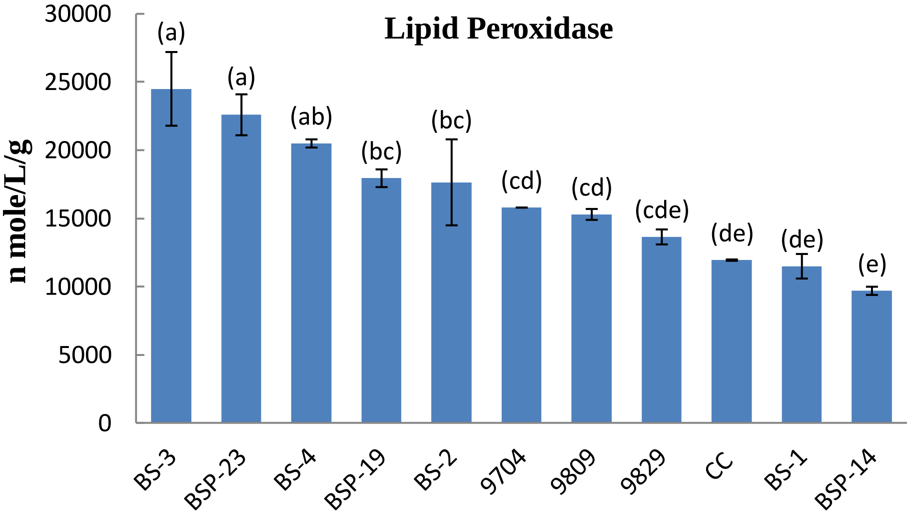


Figure: 4.16 Effect of seed bacterization of *Pseudomonas* isolates on the activity of Lipid Peroxidase (LPO) in chickpea leaves

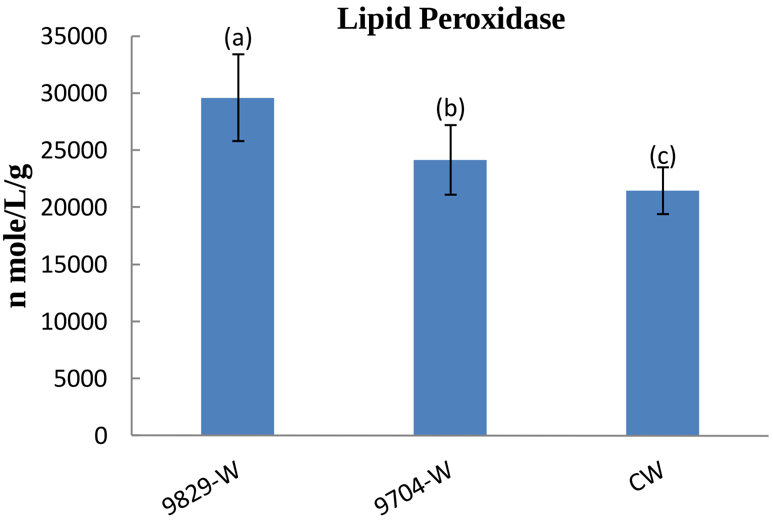


Figure: 4.17 Effect of seed bacterization of *Pseudomonas* isolates on the activity of Lipid Peroxidase (LPO) in wheat leaves

*All the treatment values are average of three replications
*All the treatments found significant at a 5 % probability level
*Means followed by a common letter are not significantly different at 5 % level
*Error bars indicate one standard error of the mean

the mechanical strength of the cell wall and inhibits fungal pathogen. The present investigation revealed that variation in levels of accumulation of TPC was consistently higher in chickpea and wheat samples derived from treatment with *Pseudomonas* isolates. In the present investigation accumulation of phenolics was higher in chickpea and wheat plants pre-treated with *Pseudomonas* isolates in comparison with control or untreated plants (Table 4.16a, 4.16b). The amount of total phenolic content (TPC) was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as ug GAE/g gallic acid equivalent using standard curve equation: $y = 0.0127x – 0.2234$, $R^2= 0.9697$. Where y is the absorbance at 725nm and x is total phenolic content in different *Pseudomonas* treated chickpea and wheat plants expressed in ug GAE/g. TPC in chickpea plant varied from 1375.75±10 to 696.96±16.04 ug GAE/g (Figure: 4.17). The maximum phenolic content was found to be 1375.75 ± 10 and 1297.92 ± 38.75 ug GAE/g in chickpea and wheat plants (Figure: 4.18) respectively treated with *Pseudomonas* isolate 9704, in comparison with control or untreated plants. All the other treatments except BSP-23 in chickpea showed increased activity of TPC. Seed bacterization with *Pseudomonas* isolates 9704 led to an increase in TPC activity of up to 30.3% and 46.27% in chickpea and wheat plants respectively. Similar results of variation in polyphenol synthesis and accumulation were also observed in leaves of chickpea leaves under the influence of different microbial treatments by Singh et al (2014). Selvaraj and Ambalavanan (2013) noticed phenol accumulation in all plants treated with biocontrol agents and had a profound effect on the accumulation of phenols in plants upon challenge inoculation with *C. gloeosporioides*. Similar results were also obtained. by Maurya and Singh (2010) in Adathoda vasica leaves. Karthikeyan et al (2006) also recorded the accumulation of phenols in coconut roots, soil-applied with biocontrol agents in challenge inoculation with *Ganoderma lucidum.*

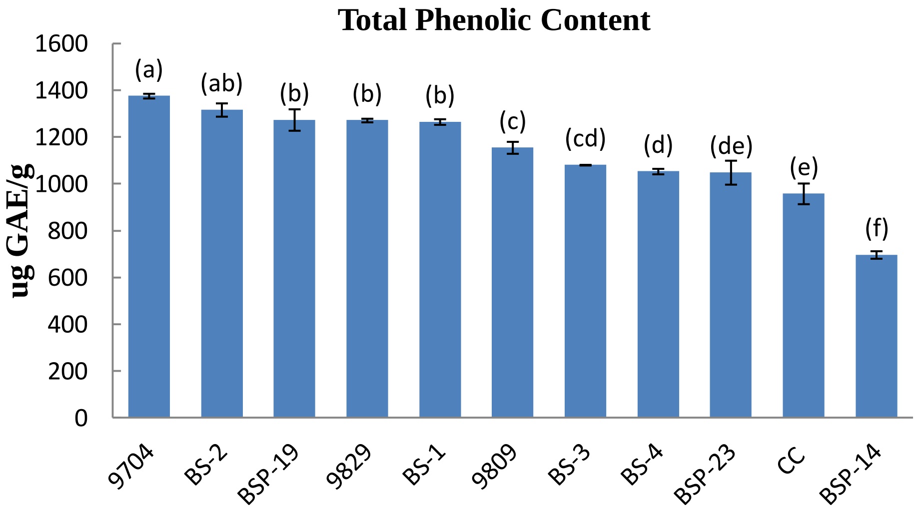


Figure: 4.18 Effect of seed bacterization of *Pseudomonas* isolates on the activity of Total Phenolic Content (TPC) in chickpea leaves

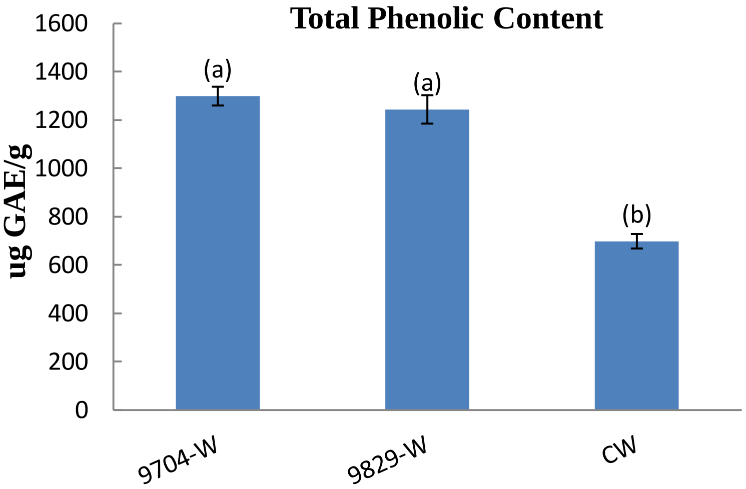


Figure: 4.19 Effect of seed bacterization of *Pseudomonas* isolates on the activity of Total Phenolic Content (TPC) in wheat leaves

*All the treatment values are average of three replications
*All the treatments found significant at 5 % probability level
*Means followed by a common letter are not significantly different at 5 % level
*Error bars indicate one standard error of the mean

4.9 Molecular characterization of fluorescent *Pseudomonas* isolates using genus and species-specific loci.

The nucleotide sequence of small-subunit (SSU) rRNA has been widely used to identification and classification of microorganisms (Woese 1987). Genomic DNA of ten *Pseudomonas* isolates was extracted as per manufacturer's instructions using HiPurA^{TM} genomic DNA purification kit (Cat. no.K3100-02) and used for amplification using various primers (Table: 3.2). 16S and 27F+1492R PCR primer sets were used for specific identification of species of ten *Pseudomonas* isolates by amplification of the nuclear rRNA gene cluster. Pioneer work of Woese (1987) points out the widespread use of 16S rRNA gene sequences for bacterial identification and taxonomy. The 16S rRNA gene sequence is about 1550 bp long and is composed of both variable and conserved regions.

In the present investigation PCR amplification of 16S rRNA bacterial gene (1500bp) using 16S and 27F+1492R primer set resulted in a specific distinguishing amplification product. All the ten *Pseudomonas* isolate resulted in a positive reaction with 16S and 27F+1492R PCR primer sets and got an amplicon size of ~1100 bp (Plate:) and ~1150 bp (Plate: 4.24, 4.25) respectively.

4.9.1 ERIC PCR based genotype analysis of *Pseudomonas* isolates

Repetitive element based PCR has been used to study the strain-specific patterns obtained from PCR amplification of repetitive DNA present in prokaryotic genomes and used as primer site for genomic DNA amplification (Proudy et al. 2008). Three families of the repetitive sequence have been studied includes REP sequence (35-40 bp), ERIC sequence (124bp), and BOX sequence (154bp) (Lupski and Weinstock 1992). Enterobacterial repetitive intergenic consensus (ERIC) or also known as intergenic repeat units (IRUs) (Sharples and Lloyd 1990) is 126bp long with a highly conserved central inverted repeat. It is located in the non-coding transcribing region on the chromosome (Waturangi et al. 2012) and synthesizes DNA sequence outward from inverted repeats (Yoke-Kqueen et al. 2013). ERIC sequences can be utilized as a primer binding site in PCR reaction to produce a fingerprint of the different bacterial genome. In the present investigation, ERIC primer sequence was used in PCR to detect

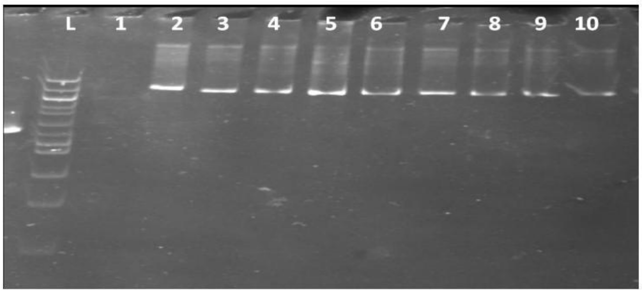


Plate: 4.24 PCR amplification of ten fluorescent *Pseudomonas* isolates generating 1100bp bands through 16S rRNA primer (L-100bp ladder)

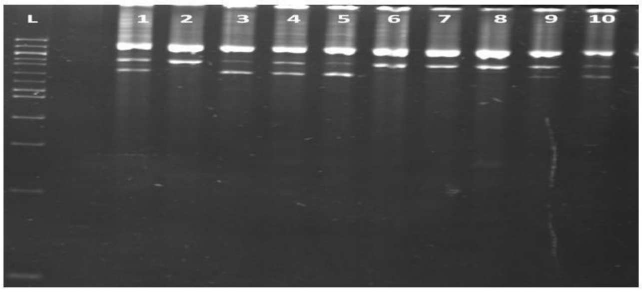


Plate: 4.25 PCR amplification of ten fluorescent *Pseudomonas* isolates generating 1150bp bands through 27+1492 primer (L-100bp ladder)

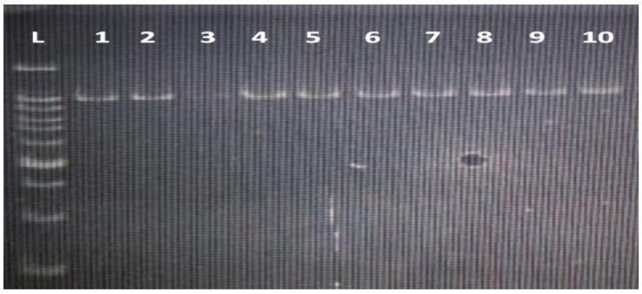
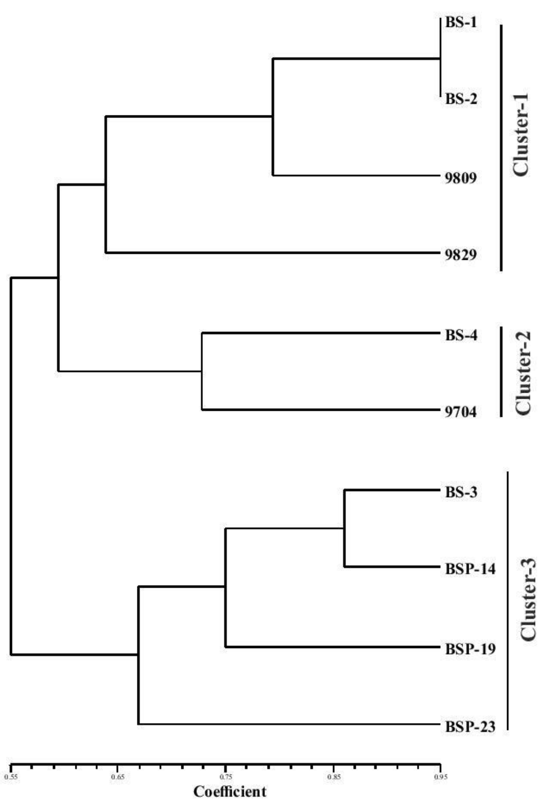


Plate: 4.26 PCR amplification of ten fluorescent *Pseudomonas* isolates generating 900bp bands through Prn A primer (L-100bp ladder)

Plate 24, 25, 26



ERIC PCR Based genotype analysis

Plate: 4.27 ERIC-PCR-based genotypic analysis of 10 *Pseudomonas* isolates. Dendrogram of 10 *Pseudomonas* isolates generated by binary matrix derived from ERIC amplicons

Plate: 4.27 ERIC-PCR-based genotypic analysis of 10 *Pseudomonas* isolates. Dendrogram of 10 Pseudomonas isolates generated by binary matrix derived from ERIC amplicons.

differences in the number and distribution of bacterial repetitive in ten *Pseudomonas* isolate genome . A high level of polymorphism was seen in PCR of 10 *Pseudomonas* isolates (Plate: 4.27). The number of bands after PCR amplification of isolates with ERIC primer varied from 4 – 10 with molecular weights between 50 and 800bp. Genetic similarity among ten *Pseudomonas* isolates varied from 0.55 to 0.95 (Plate: 4.28). Similarity data obtained with ERIC primer identified three clusters. Overall cluster analysis based on pair-wise coefficient similarity with UPGMA of ERIC- PCR resulted into 3 distinct genomic clusters at similarity coefficient 0.55, *viz.,* cluster-1 (BS-1, BS-2, 9809, 9829), cluster-2 (BS-4 and 9704) and cluster-3 (BS-3, BSP-14, BSP-19, BSP-23) isolates. Among all *Pseudomonas* isolates BS-1 and BS-2 showed 100% similar ERIC-PCR pattern as they were obtained from the same sources (*Brassica* rhizosphere soil samples). All the isolates exhibited their high degree of genetic variability and were distributed into different clusters. ERIC-PCR fingerprinting showed wide variation due to a high degree of DNA heterogeneity overall ten *Pseudomonas* isolates.

ERIC-PCR confirmed differences in repetitive elements dispersion in *Pseudomonas* genomes and a high degree of genetic variability among ten *Pseudomonas* isolates. Botelho (2001) studied *Pseudomonas* isolates from the soybean rhizosphere cultivated in Brazilian savannah were analyzed by ERIC-PCR and it was observed that a major part of them had a similar profile to the *Pseudomonas fluorescence.* Similar results were also observed by Agrawal et al (2015) studied ERIC-PCR-based genotyping analysis of 24 *P. putida* isolates and observed a high degree of DNA heterogeneity over all the 24 *P. putida* isolates.

4.9.2 In-vitro detection of antibiotic-producing *Pseudomonas* isolates using gene-specific primers

Many authors reported that fluorescent *Pseudomonas* spp. isolated from soil produce antibiotics *viz.,* pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA), and 2,4 Diacetlyphloroglucinol (Phl) that are naturally suppressive to plant diseases (Keel et al. 1996; Dutrecq et al. 1991; Fenton et al. 1992; Agrawal et al. 2015). The PCR product was fractioned by gel

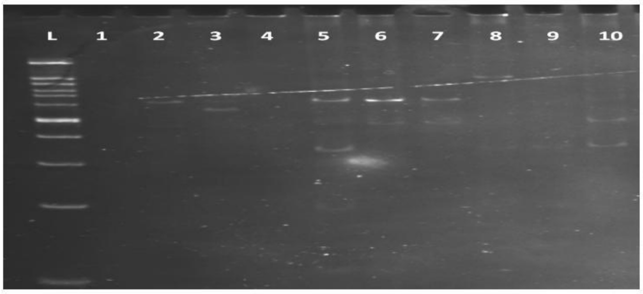


Plate: 4.28 PCR amplification of ten fluorescent *Pseudomonas* isolates generating 600 bp bands through Phl 2a-2b primer (L-100bp ladder)

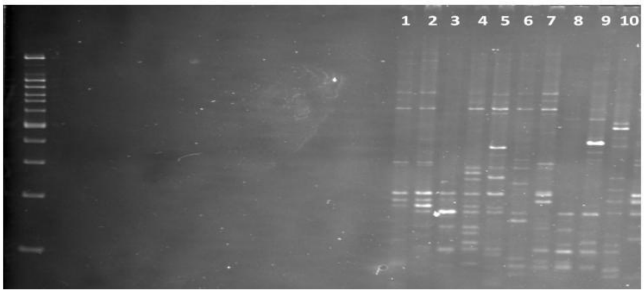


Plate: 4.29 PCR amplification of ten fluorescent *Pseudomonas* isolates generating multiple bands through ERIC primer (L-100bp ladder)

Plate 28, 29

electrophoresis in 1X TBE buffer. The gel was stained with ethidium bromide and viewed under gel documentation provided with UV light. Results obtained from PCR analysis with primer Prn AF-R (Pyrrolnitrin) amplified ~900bp fragment in all the isolates (Plate: 4.25). 2, 4 Diacetylphloroglucinol gene-specific primer Phl2a-2b amplified ~650bp fragment (Plate: 4.26) in isolates 9704, 9829, and 9809 only, faint bands were observed in BS-2, BS-3, BSP-14, BSP-19, and BSP-23. No amplification was observed with isolates BS-1 and BS-4.

Results of PCR analysis with primers of different antibiotic genes showed ten *Pseudomonas* isolates contained genes for Pyrrolnitrin and all *Pseudomonas* isolates except BS-1 and BS-4 contained the gene for 2, 4 Diacetylphloroglucinol. Antibiotic Prn-A and Phl are major determinants of biological control of soil born plant pathogens by strains of fluorescence *Pseudomonas* (Thomashow and Weller 1996). Confrontation assay was performed by *Pseudomonas* isolate 9704 against *Macrophomina phaseolina* resulted in 60% inhibition, which may be due to the production of antibiotic 2, 4 Diacetylphloroglucinol by isolate 9704. Similar results were also reported by Agrawal et al (2015) isolate P132 showed the presence of genes for Phenazine, pyrrolnitrin, and 2, 4 Diacetylphloroglucinol along with polyhydroxyalkanoate.

CHAPTER-V
SUMMARY AND CONCLUSIONS

Significant findings of the present investigation **"Field evaluation of potential fluorescent *Pseudomonas* isolates on growth and yield attributing characters and induction of antioxidant enzyme in soybean, chickpea, and wheat"** summarized as follows;

Collection and isolation of fluorescent *Pseudomonas* from soil and *Brassica* specific rhizosphere.

Rhizospheric soil of different crop plants collected from different locations of Bastar and Bilaspur districts of Chhattisgarh. Four rhizosphere soil samples were derived from *B. napus* from Zhahirabad (Telangana) and isolated fluorescent *Pseudomonas* following serial dilution method on King's B agar media. Single bacterial colonies of fluorescent *Pseudomonas* were detected under the UV light (366nm) and further purified by streaking on to the Kings'B media plates. Ten isolates emitting very strong fluorescence on Kings B media out of 88 were selected for further investigation.

Metabolic profiling and sequence analysis of selected fluorescent *Pseudomonas* isolates

Standard data for selected for nutritional and general phenotypic characteristics as given in Bergey's Manual of Systematic Bacteriology (Palleroni 1984) for selected fluorescent *Pseudomonas* species (*P. aeruginosa, P. fluorescens* bv. I, bv. II, bv. III, bv. IV, bv. V, *P. putida* biovar A and biovar B) was utilized to compare the metabolic profiles resolved by 10 isolates used in the present investigation. Based on the standard data for selected nutritional and general phenotypic characteristic, of *P. aeruginosa* expressed70% similarity with the 10 isolates used in the present investigation which indicated that standard data set also drive the component of species identification. Based on the phylogenetic affinities of the 16S rRNA DNA sequence derived from the isolates BS-1, BS-2, BS-4, 9704, 9829, 9809, and BSP-19 species identity is delineated as *P. aeruginosa* and for BS-3 as *P. chlororaphis* sub sp *chlororaphis* and BSP-14 and BSP-23 as *P. mesoacidophilla.*

Biochemical characterization of fluorescent *Pseudomonas* isolates.

All the ten *Pseudomonas* isolates screened qualitatively and quantitatively for siderophore production, ACC deaminase, IAA production, and phosphate solubilization. All the isolates produced a varying level of ACC, siderophore, IAA, and were able to solubilize phosphate. Among 10 *Pseudomonas* isolates, isolate 9704 was identified as the highest phosphate solubilizer (357.5µg/ml). BSP-23 (28.08 µg/ml) was identified as the highest IAA producer. BSP-19 (79.71%) was the highest siderophore producer. Fluorescent *Pseudomonas* isolates BS-1, BS-4, 9809, BSP-14, and BSP-19 were identified to possess ACC deaminase producing ability following qualitative analysis.

In-vitro antagonistic activity by fluorescent *Pseudomonas* against *Macrophomina phaseolina*

Confrontation assays were performed using selected 10 fluorescent isolates against *M. phaseolina* expressed varying levels of inhibition (47.2% to 61.1%) and identified potential fluorescent *Pseudomonas* isolates (9704 and 9809).

In-vitro infection model for *Macrophomina phaseolina* causing soybean charcoal rot

In-vitro semi-artificial experimental set-ups offered several advantages but lack the root-soil interface. Interlinked multi-component complexity of the rhizosphere required experimental designs and at present, there is no described methodology for *in-vitro* interaction involving root-soil interface in semi-artificial experimental setups. In the present study, we report, a method to raise plantlets of different crops in modified Petri plates/rings and described methodology for *in-vitro* interaction involving root-soil interface in semi-artificial experimental setups. We also monitored the colonization of fluorescent *Pseudomonas* in soybean roots following the root imprinting method. Compared to the early steps of *M. phaseolina* infection progress in the presence-absence of fluorescent *Pseudomonas* and proposed some reasons for differences observed.

Effect of seed bacterization with *Pseudomonas* isolates on the germination and growth of soybean seedlings

Plant growth-promoting abilities of ten *Pseudomonas* isolates were evaluated in soybean (CG SOYA-1), chickpea (INDIRA CHANA-1), and wheat (AMBER WHEAT) cultivars following seed treatment. The effect of seed bacterization with ten *Pseudomonas* isolates on the germination and growth of soybean seedlings were studied. Following seed treatment with ten *Pseudomonas* isolates significantly enhanced both germination and growth of soybean seedlings. Early germination of soybean seeds treated with *Pseudomonas* isolates was recorded. Besides, the highest root and shoot growth was recorded on the 7th day in soybean seeds treated with isolate 9704.

Plant growth-promoting response in soybean (CG SOYA-1) following seed bacterization with fluorescent *Pseudomonas* isolates

Pot and field evaluation of ten *Pseudomonas* isolates revealed improved pant growth-promoting parameters in soybean when compared to control. Most of the studied traits such as shoots length, root length, number of branches, and number of filled pods were significantly different at the 5 % probability level. Pot experiment revealed that shoot length was highly stimulated by seed inoculation with *Pseudomonas* isolate (9704). Bundle weight, straw yield, and grain yield in soybean plants treated with *Pseudomonas* isolates were markedly increased in comparison to control. Increased grain yield was recorded in soybean plants treated with *Pseudomonas* isolate 9704 which led to a 38.67 % increase in grain yield.

Plant growth-promoting response in chickpea (INDIRA CHANA-1) following seed bacterization with fluorescent *Pseudomonas* isolates.

Plant growth-promoting response in chickpea following seed bacterization with fluorescent *Pseudomonas* isolates revealed most of the studied traits such as shoots length, root length, number of branches, and root volume were significantly different at the 5 % probability level. Pot experiments revealed that a distinct difference was seen in the root volume of chickpea plants treated with *Pseudomonas* isolates (BS-2) which led to a 61.53 % increase over control. Bundle weight, straw yield, and grain yield in chickpea plants treated with *Pseudomonas* isolates were markedly increased in comparison to untreated or control. The highest grain yield was recorded in chickpea plants treated with *Pseudomonas* isolate BS-4 which led to a 37.9 % increase in grain yield over control.

Plant growth-promoting response in wheat (AMBER WHEAT) following seed bacterization with fluorescent *Pseudomonas* isolates.

In a field experiment, all the parameters attributing growth and yield in amber wheat were influenced by two *Pseudomonas* isolate treatments. A significant increase in shoot length, shoot fresh and dry weights, grain numbers per spike in each tiller in both the treatments over control. Bundle weight, straw yield, and grain yield in wheat plants treated with *Pseudomonas* isolates were markedly increased and the highest grain yield was recorded in wheat plant treated with *Pseudomonas* isolate 9829 which led to a 15.5% increase in grain yield.

Effect of seed bacterization with *Pseudomonas* isolates in chickpea and wheat leaves contents of superoxide dismutase (SOD), peroxidase (POX), phenylamine ammonia-lyase (PAL), lipid peroxidase (LPO), polyphenol oxidase (PPO), and total phenolic content (TPC).

Seed bacterization with *Pseudomonas* isolates also induced accumulation of high levels of antioxidant enzymes (SOD, POX, PAL, LPO, PPO, and TPC) in flag leaves of chickpea and wheat plants when compared to control. Different antioxidant enzymes were assayed in leaves of chickpea plants treated with 10 *Pseudomonas* isolates. SOD (37.7%), POX (17.78%), PAL (1.65%), LPO (51.2%), PPO (42.9%) and TPC (30.3%) increase over control. Similarly, different antioxidant enzymes were assayed in leaves of wheat plants treated with 10 *Pseudomonas* isolates. POX (3.18%), PAL (2.65%), LPO (27.53%) and TPC (46.27%) increase over control.

Molecular characterization of fluorescent *Pseudomonas* isolates using genus and species-specific loci.

Molecular characterization of 10 *Pseudomonas* isolates was done by using 16S and 27F+1492R, antibiotic gene-specific primers PrnA (pyrrolnitrin), 2, 4 diacetyl phloroglucinol (Phl), and ERIC based genotype analysis. PCR amplification of the 16S rRNA bacterial gene (1500bp) using 16S and 27F+1492R primer set resulted in an amplicon size of ~1100bp and ~1150bp respectively. ERIC-based genotype analysis of 10 *Pseudomonas* isolates showed a high degree of heterogeneity among ten *Pseudomonas* isolates and was distributed into three distinct genomic clusters with a similarity coefficient of 0.55. Ten *Pseudomonas* isolates showed positive results with antibiotic gene-specific primers PrnA (pyrrolnitrin) and Phl (2, 4 diacetyl phloroglucinol) and amplified product size of ~900bp and ~650bp respectively.

CONCLUSION

1. To augmentation of the beneficial effects of fluorescent *Pseudomonas*, seed treatment was the simple and cost-effective method for the delivery of bio-inoculant.
2. Bacterial isolates (9704, BS-4, and 9829) consistently, over multiple locations, improved seed germination, reduced seedling mortality, improved plant growth, and yield of Soybean Chickpea and wheat. These isolates also stimulated enhanced antioxidant activity in chickpea and wheat
3. Plant growth-promoting effects and increase in grain yield of fluorescent *Pseudomonas* isolates were crop-specific (soybean (9704-38.67%), chickpea (BS-4-37.9%), and wheat (9829-15.5%)
4. Phylogenetic affinities of the 16S rRNA DNA and ITS27 + ITS1492 gene sequences resolved to isolate BS-3 as *P. chlororaphis* sub sp *chlororaphis* and BSP-14 and BSP-23 as *P. mesoacidophilla.*
5. In the present study, we report, a method to raise plantlets of different crops in general and soybean in particular in modified Petri plates and described methodology for *in vitro* interaction (bi and tri-partite interaction) involving root-soil interface in semi-artificial experimental set-ups.
6. Traceability of the infection process of *M. phaseolina* on soybean (which was otherwise difficult in the root-soil interface are limited owing to belowground nature, soil opacity, and complexity) was possible with our proposed technique (modified Petri plates).

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RESUME

Name of Student	Rathod Parashuram
Date of birth	18-05-1991
Present address	Swami vivekanand boys hostel, zora, Raipur, CG
Phone	9705905982
E-mail	parshunaik25@gmail.com
Permanent Address	H.No 7-14 Dattagiri colony, zhahirabad, Sangareddy (dist), Telangana 502220

Academic Qualification

Degree /Diploma	Year of Passing	Board University
SSC	2007	SSC (A.P)
INTERMDIATE	2009	BIE
B.Sc. (Ag.)	2015	P.J.T.S.A.U
M. Sc. (Ag.)	2017	I. G. K. V
Ph.D	2020	I. G. K. V

Professional Experience (If any): No

Membership of Professional Societies (If any): YES

Awards / Recognitions (If any): YES

Publications (If any): Two

Signature