

**“Development and evaluation of *Pseudomonas* based
bioformulation for disease control and growth
enhancement of *Zea mays* L.”**

Thesis
Submitted to
Babasaheb Bhimrao Ambedkar University
Lucknow

BABASAHEB
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•LUCKNOW•
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2016

DEDICATED
TO
MY GRANDFATHER

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CERTIFICATE

This is to certify that the thesis titled “**Development and evaluation of *Pseudomonas* based bioformulation for disease control and growth enhancement of *Zea mays* L.**” submitted by **Mr. Jitendra Mishra** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

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

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DECLARATION

This is to certify that I have worked on the research thesis entitled “**Development and evaluation of *Pseudomonas* based bioformulation for disease control and growth enhancement of *Zea mays* L.**”. The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree.

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Jitendra Mishra

LIST OF ABBREVIATIONS

%	Percent
° C	Degree Celsius
° E	Degree East
° N	Degree North
µL	Micro Liters
µS/cm	Micro-Siemens per Centimeter
ABA	Abscisic Acid
ACC	1-Aminocyclopropane-1-Carboxylate
BLAST	Basic Local Alignment Sequence Tool
BNF	Biological Nitrogen Fixation
bp	Base Pairs
BSI	Bureau of Indian Standard
CAGR	Calculated Annual Growth Rate
CAS	Chrome Azurol S
CFU	Colony Forming Unit
CIMMYT	International Maize and Wheat Improvement Center (Spanish Acronym)
CLPs	Cyclic Lipopeptides
cm	Centimeters
CMC	Carboxymethylcellulose
d	Days
DAPG	2,4-Diacetylphloroglucinol
DAS	Days After Sowing
DMRT	Duncan's Multiplicity Range Test
DNA	Deoxy Ribonucleic Acid
EC	Electrical Conductivity
EC	Esophageal Cancer
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization of The United Nations
FAOSTAT	Fao Statistical Databases
Fe	Iron
FT-IR	Fourier Transmission Infra-Red
g	Gram
GAs	Gibberellins
GDP	Gross Domestic Product
h	Hours
ha	Hectare
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethylammonium
IAA	Indole-3-Acetic Acid
ISR	Induced Systemic Resistance
K	Potassium
KB	King's B Medium
kg	Kilogram
KSB	Potassium Solubilizing Bacteria
LEM	Leukoencephalomalacia
m	Meter

m	Minutes
mg	Milligram
Mha	Million Hectares
ml	Mili Liters
MMT	Million Metric Tons
MR-VP	Methyl-Red and Voges-Proskauer
N	Nitrogen
N₂	Dinitrogen
nm	Nano Meter
OF	Oxidative-Fermentative
P	Phosphate
PCA	Phenazine-1-Carboxylic Acid
PDA	Potato Dextrose Agar
PDC	Phenazine-1,6-Dicarboxylic Acid
PES	Pulmonary Edema Syndrome
PES	Pulmonary Edema Syndrome
PGP	Plant Growth Promoting
PGPR	Plant Growth Promoting Rhizobacteria
PHB	Poly-P-Hydroxybutyrate
PIPES	Piperazine-N, N'-Bis (Ethane Sulfonic acid)
PSB	Phosphate Solubilizing Bacteria
PSI	Phosphate Solubilization Index
psi	Pounds per Inch Square
ROS	Reactive Oxygen Species
rpm	Rotation per Minutes
rRNA	Ribosomal Ribonucleic Acid
s	Seconds
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography
USA	United States of America
UV	Ultra Violet
w/v	Weight by Volume
WHO	World Health Organization
Zn	Zinc
ZSI	Zinc Solubilization Index
β	Beta

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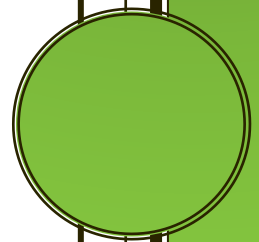
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INTRODUCTION



1. INTRODUCTION

Cereal crops are the principal source of calories for the majority of the world population. Cereals like wheat, rice and maize are the most important food commodities. Maize (*Zea mays L.*) is a major cereal crop and due to its high yield potential among the cereals it is globally known as Queen of cereals. Maize grains contain high content of carbohydrates, fats, proteins and some of the important vitamins (C, E, K, B₁, B₂, B₃, B₅, B₆, folic acid, selenium, N-p-coumaryl tryptamine, and N-ferrulyl tryptamine) and minerals such as potassium (**Figure 1**). The B-complex vitamins are good for skin, hair, heart, brain, and proper digestion, prevent the symptoms of rheumatism. However, vitamins A, C and K together with beta-carotene and selenium helps to improve the functioning of thyroid gland and immune system (Shah et al. 2016). Maize oil contains 14% saturated fatty acids, 30% monounsaturated fatty acids, and 56% polyunsaturated fatty acids. The refined maize oil contains linoleic acid 54–60%, oleic acid 25–31%, palmitic acid 11–13%, stearic acid 2–3% and linolenic acid 1% (CRA 2006). Several million people, particularly in the developing countries, derive their protein and calorie (15 to 56%) requirements from maize (FAO 1992). Maize silk contains various ingredients essential for our diet such as maizenic acid, fixed oils, resin, sugar, mucilage, salt, and fibers (Kumar and Jhariya 2013). Maize is also an essential source of numerous phytochemicals such as carotenoids, phenolic compounds, and phytosterols (Jiang and Wang 2005; Lopez-Martinez et al. 2009).

Maize is grown throughout the world in various agro-climatic zones. This cereal crop not only plays a major role in the diet of millions of humans but also constitutes a basic element of animal feed and raw material for the manufacture of many industrial products (Rautaray et al. 2003; Lee et al. 2013). It is the third leading crop of the world after rice and wheat (Sandhu et al. 2007). The crop is cultivated in 160 countries having a wide diversity of soil, climate, biodiversity and management practices and contributes 36 % of the global

Nutritional Value of corn (per 100g)	
Carbohydrate	71.88 g
Protein	8.84 g
Fat	4.57 g
Fiber	2.15 g
Riboflavin	0.10 mg
Thiamine	0.42 mg
Vitamin C	0.12 mg
Amino acids	1.78 mg
Sodium	15.9 mg
Phosphorus	348 mg
Sulfur	114 mg
Copper	0.14 mg
Magnesium	139 mg
Potassium	286 mg
Iron	2.3 mg
Calcium	10 mg

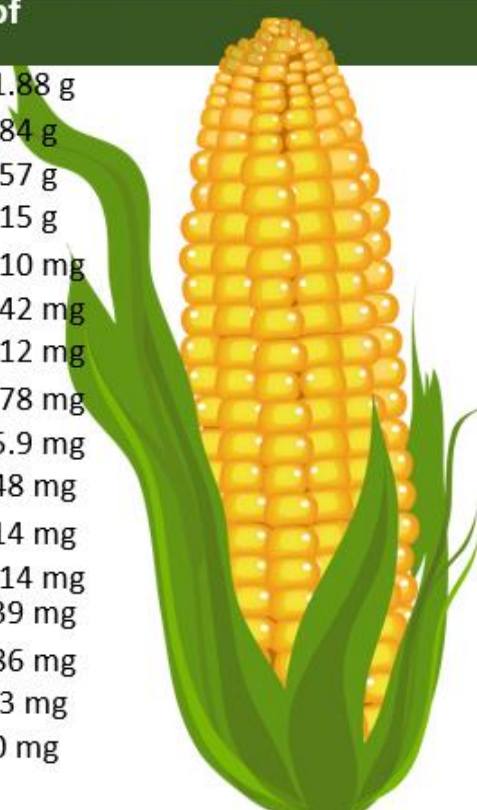


Figure 1. Nutritional value of per 100 g of edible portion of maize
(Source: Modified from Shah et al. 2016)

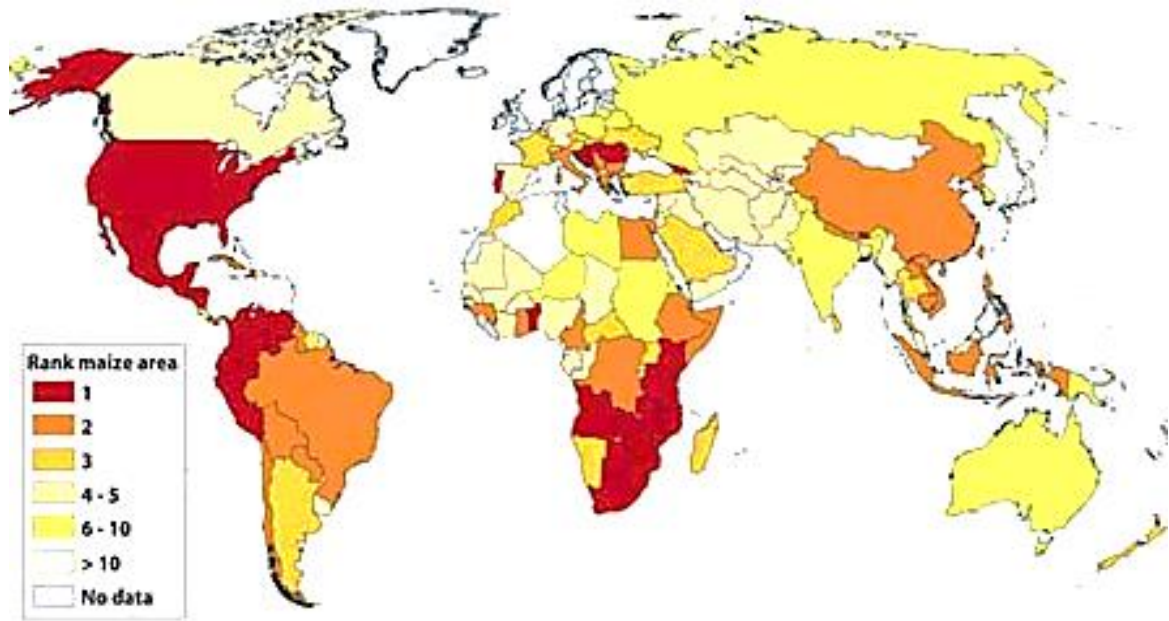


Figure 2. Maize area sown worldwide according to their rank (Source: www.maize.org)

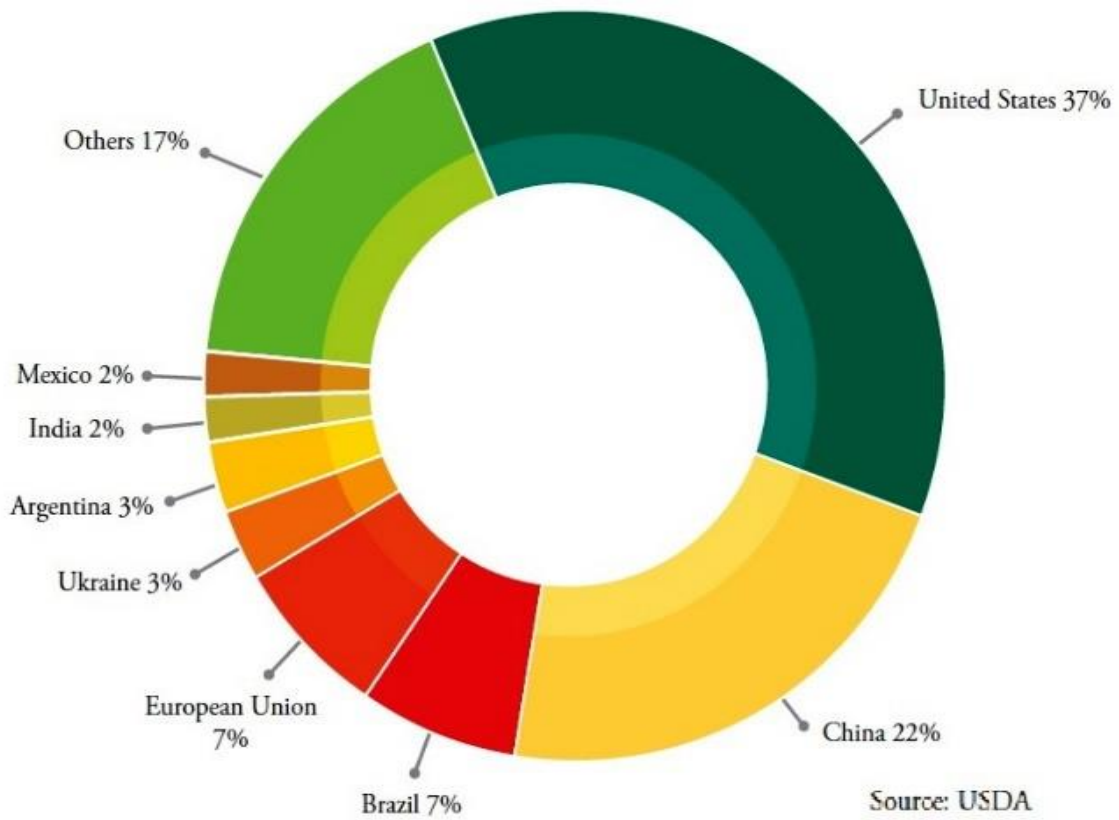


Figure 3. Top Maize Producing Countries in year 2013-14
(Source: Adopted from India Maize Summit 2014)

grain production (**Figure 2**) (Plessis 2003). However, recent data showed that at global level, area under maize cultivation has increased at a calculated annual growth rate (CAGR) of 2.2% i.e. from 146 million hectares (Mha) in 2004-05 to 177 Mha in 2013-14 (India Maize Summit 2014). Global maize production has grown at CAGR of 3.4 % over the last ten years from 716 million metric tons (MMT) in 2004-05 to 967 MMT in 2013-14. The United States of America (USA) is the largest producer followed by China and Brazil. USA contributes 37 % of the total (**Figure 3**) whereas, the average productivity in India is 2.43 trillion hectares per year). In India, also maize is the third most important food crop after rice and wheat. Maize production in India has grown at a CAGR of 5.5 % over the last ten years from 14 million metric tons in 2004-05 to 23 million metric tons in 2013-14 (India Maize Summit 2014).

In last few years, maize production in India has increased but at the same time is also suffering yield losses due to various biotic stresses including phytopathogens (Shekhar and Kumar 2010). It has been observed that in various parts of the country control measures applied for its disease control are not up to the mark and more often involved chemicals/pesticides (Rautaray et al. 2003; Lee et al. 2013; Tiwari et al. 2013). Amongst reported diseases of maize, soil-borne diseases are most destructive (Koehler 1960). The eradication of soil-borne diseases is seemingly more challenging as the pathogens exclusively reside in the soil and start damaging the plant by penetrating the root or basal stem (Browning et al. 1969). Pathogen interact with plant roots and soil constituents at the root-soil interface, where root exudates and decaying plant material provide carbon for the heterotrophic biota (Barea et al. 2005; Bisseling et al. 2009). According to Oerke (2005), worldwide losses in maize due to diseases (not including insects or viruses) were estimated to be about 9% in 2001–2003 and further varied significantly by region with estimates of 4% in northern Europe and 14% in West Africa and South Asia

(www.cabicompendium.org/cpc/economic.asp). According to the international maize and wheat improvement center, known by its Spanish acronym (CIMMYT), maize suffers from about 110 diseases mainly caused by fungi, bacteria, and viruses (Harinder et al. 2014). In India, there are records of about 35 of them caused by fungi, bacteria and viruses that account for 13.2% of the crop loss in economic terms (Dhillon and Prasanna 2001). Fungal pathogens like *Aspergillus flavus* (Aspergillus ear and kernel rot), *A. glaucus* (minor ear rots), *A. niger* (minor ear rots), *Rhizopus stolonifer*, *Lasiodiplodia theobromae* (Black kernel rot) *Macrophomina phaseolina* (seed rot, seedling blight), *Penicillium* spp. (Penicillium ear rot, Blue eye, Blue mold) are of great concern. However, diseases caused by *Fusarium* spp. such as Fusarium ear rot (*Fusarium moniliforme*, *F. graminearum*, *F. verticillioides*, *F. subglutinans* and *F. proliferatum*), Gibberella ear rot (*F. graminearum*), Fusarium stalk rot (*F. verticillioides*), Gibberella stalk rot (*F. graminearum*) and Fusarium root rot (*F. graminearum* and *F. verticillioides*) and seedling blight (*F. graminearum* and *F. verticillioides*) have been considered as most destructive (Munkvold and O'Mara 2002; Rodriguez-Brljevich et al., 2010; Reyes-Velázquez et al. 2011).

Ear rot and charcoal rot disease are more prevalent in different parts of the country and responsible for grain loss (Shekhar and Kumar 2010). The general observable symptoms of ear rot diseases are the production of fungal growth in tan or brown color at the tip of the ear (**Figure 4**) (Munkvold 2003). The fungus is transmitted vertically and horizontally to the next generation of plants via clonal infection of seeds and plant debris. It also exists as an endophyte and systematically colonizes kernels and infects the emerging seedlings, maturing plant, and the new kernel (Bacon and Hinton 1996). The distributions of Fusarium ear rot pathogens are related to environmental conditions such as temperature, humidity, light intensity and wind (Bottalico 1998). This is most common fungi associated with basic human and animal dietary samples and produces several mycotoxins as beauvericin, fumonisin,



a. Pink, red or deep red growth of fungus at the ear tip



b. White fungal growth and the "starbursting" on the kernels



c. Bird or worm damage corn cob (see arrow) provide route of entry to fungus

Figure 4. Symptoms of Fusarium ear rot in maize plant

fusaproliferin and moniliformin (Logrieco et al. 2002). These toxins affect 25% of the world food crops, especially maize and pose a potential threat to humans and animals (Weidenböner 2001). In India, *F. moniliforme* and *F. semitectum* have been reported to be widespread in Uttar Pradesh, Punjab and Rajasthan (Lal and Dwivedi 1982). For controlling the disease, farmers usually apply synthetic fungicides to the plants. However, these treatments have major side effects. They have led to the appearance of environmental contamination (Jacobsen and Backman 1993). This also has increased the development of fungicide resistance amongst the pathogens (Moënné-Loccoz et al. 1998).

Every year soil borne pathogenic fungi of the genus *M. phaseolina* cause severe loss to various crops including cereals, legumes and vegetables (Su et al. 2001). *M. phaseolina* causes seedling blight, root rot and charcoal rot in more than 500 crop and non-crop species (Smith and Carvil 1977). In legumes especially in peanut, it causes seed and seedling rots, wilt, root and stem rots, leaf spots and rotting of developing pods and seed whereas in soybean, early maturation, chlorosis and incomplete pod filling are more common. In cereals such as in corn, causes charcoal rot during hot and dry conditions (Su et al. 2001). key sign of charcoal rot can be identified by gray or black discoloration in the inside the rind due to the presence of many tiny, black sclerotia (**Figure 5**). These *microsclerotia* are the primary source of infection and may persist within the soil up to three years (Dhingra and Sinclair 1977).

Approaches which are eco-friendlier and give assurance of sustainable production of the crop are of prime importance. Soil microorganisms with plant beneficial activities assist in conventional agriculture practices by enhancing yields and protection from phytopathogens (Antoun and Pre´vost 2005) Rhizospheric bacteria are of much interest in managing phytopathogens in eco-friendly manner. Plant growth promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth to describe soil bacteria and enhance



a. The very tiny black sclerotia on the vascular strands of the shredded pith (Source: www.pioneer.com)



b. Stunted growth and stem lodging in plant

Figure 5. Symptoms of Charcoal rot in maize plant

plant growth (Kloepper and Schroth 1978). Later several workers also found that soil bacteria inhabiting around/on the root surface are directly or indirectly involved in promoting plant growth and development via production and secretion of various metabolites (Kapulnik 1996; Lazarovit and Nowak 1997; Griffiths et al. 1999; Sturz and Nowak 2000; Vessey 2003; Bashan et al. 2004; Fernando et al. 2006; Fatima et al. 2009; Beneduzi et al. 2012; Ahemad and Kibert 2014). The recognition of PGPR as a group of beneficial plant bacteria has become useful for sustainable agriculture. Commercial applications of PGPR have been tested and found to be very successful (Arora et al. 2016).

A vast array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported as PGPR (Okon and Labandera-Gonzalez 1994; Glick 1995; Egamberdiyeva and Hoflich 2004; Ma et al. 2011; Wani and Khan, 2010; Beneduzi et al. 2012; Ahemad and Kibert 2014; Prathap and Ranjitha 2015; Goswami et al. 2016). PGPR help in plant growth and health promotion mainly by two means (Lugtenberg and Kamilova 2009). One aspect involved processes which are directly beneficial to plant such as atmospheric fixation of nitrogen (N), solubilization of inorganic phosphate (P), potassium (K), zinc (Zn) and potassium minerals, sequestration of iron (Fe) and most important is synthesis of phytohormones (Tank and Saraf 2010; Ahemad and Khan 2012; Sivasakthi et al. 2014). However, indirect aspect of plant health management are the activities by which PGPR prevent the deleterious effect of one or more phytopathogens (Compant et al. 2005; Fernando et al. 2006; Fatima et al. 2009). Several genera of PGPR are reported which have shown both direct and indirect activities to enhance plant growth (Arora et al. 2013; Ahemad and Kibert 2014).

Research has already proven the success of bacterial biocontrol agents in the suppression of fungal diseases (Inbar and Chen 1994) and species of *Bacillus*, *Serratia* and

Pseudomonas, have proven to be the best ones. Since last few years, biocontrol potential of fluorescent pseudomonads against phytopathogen fungi remains a matter of immense research (Varivarn et al. 2013). Abundance and prevalence of the fungi in agricultural soils is very common and make them an important group of PGPR. Biocontrol potential of fluorescent pseudomonads is due to their ability to colonize the surfaces and internal tissues of roots and stems at high densities (Humphris et al. 2005). Apart from this they compete with other deleterious soil microbes for niche and nutrients and produce various secondary metabolites (Bakthavatchalu et al. 2012). The main mode of action of fluorescent pseudomonads as biocontrol agents includes antibiosis and induced systematic resistance (ISR). Antibiosis is an attractive and powerful mode of action adopted by various bacteria and used in biocontrol of plant diseases (Mercado-Blanco and Bakker 2007). Fluorescent pseudomonads produce many secondary metabolites which not only are effective against wide range of phytopathogenic fungi but also work against oomycetes, and bacteria (Haas and Keel 2003; Weller et al. 2007). These secondary metabolites are helpful in the control of several fungal diseases (Ganeshan and Kumar 2006). Antibiotics produced by fluorescent pseudomonads are divided mainly into six classes, i.e., phenazines, phloroglucinols, pyoluteroin, pyrrolnitrin, cyclic lipopeptides (CLPs) and hydrogen cyanide (HCN), possessing different modes of action against various bacteria and fungi causing plant diseases (Haas and Defago 2005).

Fluorescent pseudomonads capable of affecting plant growth both directly and indirectly have been very useful in increasing agriculture productivity (Panpatte et al. 2016) and research has shown that various crops treated by inoculum based on *Pseudomonas* not only enhanced crop productivity but also may minimize the use of synthetic chemicals (Adesemoye et al. 2009). Although for large-scale use, their mass production in the form of appropriate bioformulations has been started but still it is in infancy, and novel techniques

are required for state of the art production of bioinoculant. Typically, any formulation is consisting of a mixture of an active ingredient in a formulated product with inert (inactive) substances. Arora et al. (2010) define the term bioformulation to preparations of the microorganism(s) that may be partial or complete substitute for chemical fertilization/pesticides. Recently Arora and Mishra (2016) provided insight on future bioformulations and defined them as “amalgamation of the plant growth promoting microbe(s), a carrier and additives which aid the microbe in one way or the other. These additives can be the metabolite(s) or nutrients (for microbes or plants). Metabolites can be in the form of protectants, adjuvants, attractants, stimulants, anti-microbials or precursors of biological origin”. Currently, both solid and liquid bioformulations are being used in agroecosystems as an alternative to chemicals. Unfortunately, in-vitro plant growth promoting (PGP) and biocontrol traits of fluorescent pseudomonads have not consistently deciphered to success in field applications. For this, understanding the various components by which pseudomonads persevere and contend in soil would be useful in enhancing the effectiveness of formulation.

It is estimated that the demand for maize in developing countries will beat demand for wheat and rice by the year 2020 (Pingali and Pandey 2001). However, for growers, there is the continuous pressure of increasing corn productivity to fulfill the rising demand for maize in a sustainable manner. But the key issues which are currently affecting the production are the application of the huge amount of fertilizers and pesticides. Data indicates that in various agroecosystems, application of at least 60-120 kg N, 40-60 kg P and 40 kg K are required for enhancing productivity and similarly approximately 1.5 kg of fungicide for pretreatment of per kg of seed (for eradicating seed borne diseases). In India, maize can be grown in tropical, subtropical and temperate climates which cover all three seasons: rainy (Kharif), winter (rabi) and summer spring (zaid). For growing maize, farmers are more dependable on

synthetic chemicals for increasing yield and securing their crops from seed borne diseases. Although the application of PGPR based bioformulation for increasing productivity and eradicating diseases caused by phytopathogens has been in practice, in relation to maize, no products/bioformulations are currently available which provide enhanced growth and disease eradication. There is a complete lack of tailormade formulations which can tackle the issues of productivity and phytopathogens related to maize. Every year losses occur in quantity and quality of maize grains caused by phytopathogen such as *F. moniliforme* and *M. phaseolina*. Although PGPR, including fluorescent pseudomonads, are well documented as effective agents to enhance and protect plants in lab conditions but field application of these bioinoculants are still in nascent stage. Negligible reports are available for management of ear and charcoal rot of maize by biological means in field conditions. Keeping all these issues in mind and also the importance of the importance of maize following objectives were selected for the present study (also given in **Figure 6**):

- Isolation of fluorescent Pseudomonads with plant growth promoting traits from diverse conditions and rhizosphere (including maize rhizosphere)
- To check inhibitory activity of selected isolates against phytopathogens: *F. moniliforme* (ear rot) and *M. phaseolina* (charcoal rot)
- Determination of mechanism of action of selected biocontrol agent against the phytopathogens
- To monitor the biocontrol property of fluorescent *Pseudomonas* under *in vitro* and *in vivo* conditions along with its growth promotion on maize
- Development of bioformulation for growth promotion and disease management of maize
- Checking the bio-formulation in field trials

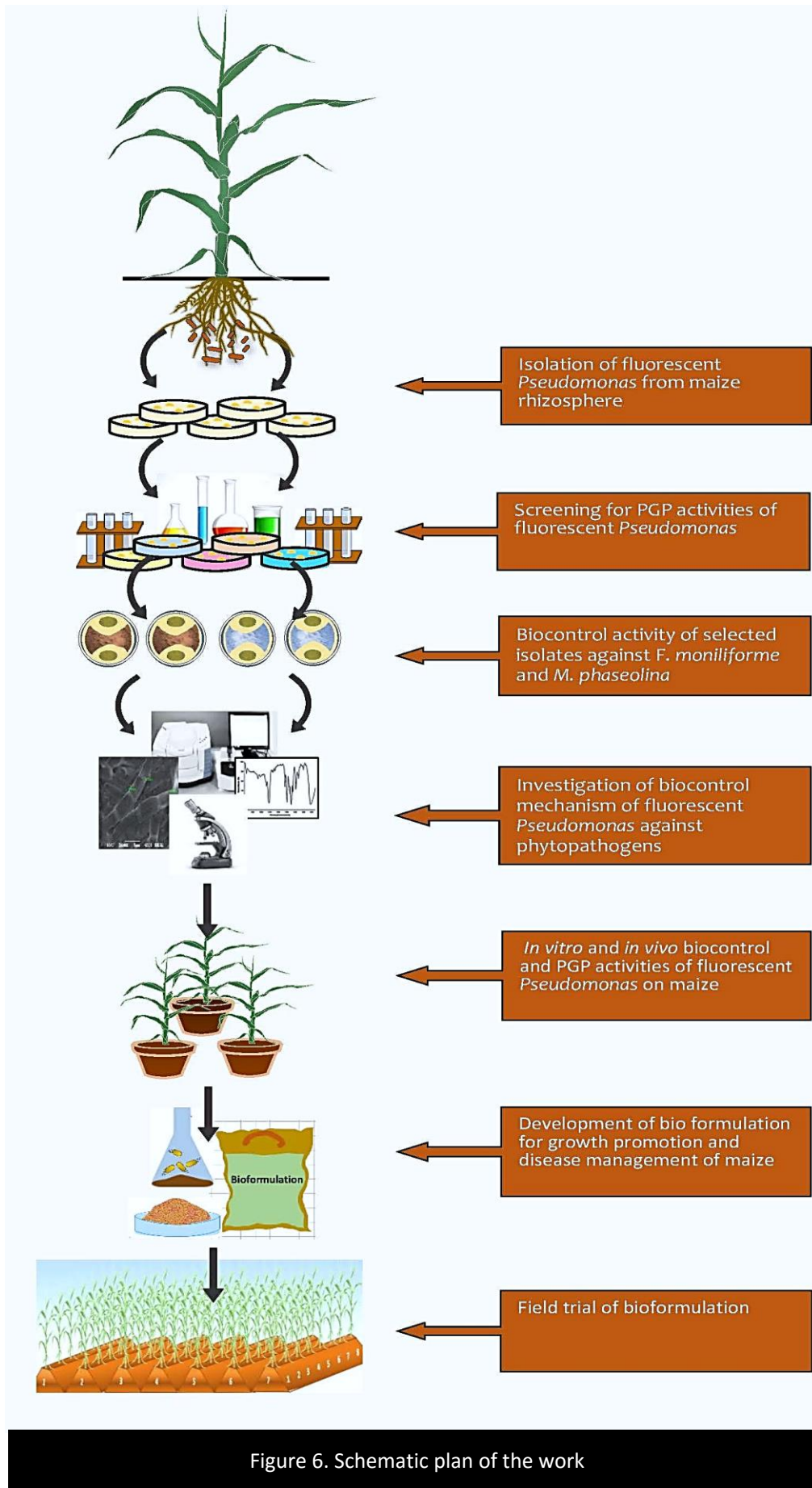
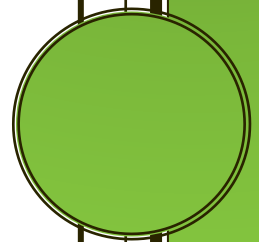


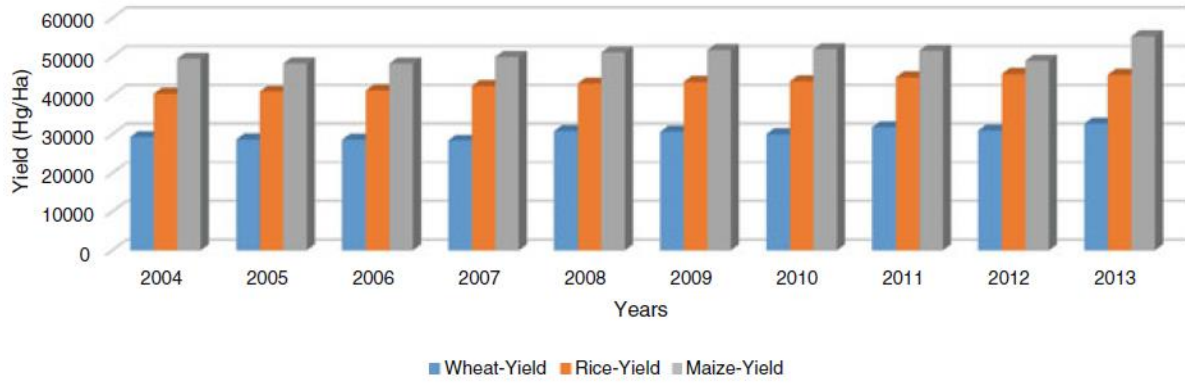
Figure 6. Schematic plan of the work

REVIEW OF LITERATURE

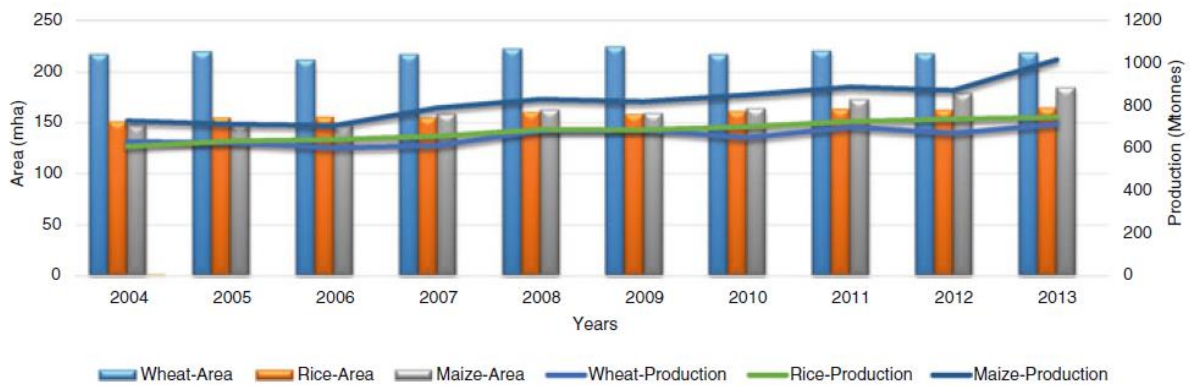


2. REVIEW OF LITERATURE

The top seven cereal crops, namely maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum* sp.), barley (*Hordeum vulgare*), sorghum (*Sorghum* sp.), oat (*Avena sativa*), and millets provide approximately two-thirds of the world food supply (Borlaug 2002). Amongst them, the big three, rice, wheat and maize are currently grown in a large number of countries, and no other cereal crops are as universally important and widely dispersed. Data indicates that cultivated area of wheat is relative more in comparison to other cereals, but yield and production of maize is more than wheat and rice (**Figure 7**) (FAOSTAT 2013). The crop has multidisciplinary uses, but its third rank as a food crop is not primarily only because of human consumption but due to its many other uses. The crop is being utilized in making more than 3,500 products used as human food, biofuel and livestock feed (**Figure 8**). Maize is an annual grass that belongs to Poaceae family. Its plants are tall (1–4 m) with broad (5–10 cm) and long leaves (50–100 cm) and extensive fibrous root system (**Figure 9**). Maize usually forms a single ear (or cob) having about 300 to 1,000 kernels on it. Origin point of maize have been a matter of debate for archaeologists and anthropologists, but all have agreed for some time that it was first domesticated in Mesoamerica (Mexico and Central America) around 8000 BC (Davidson 2006). A wild grain known as teosinte (still grows in some parts of Mexico, Guatemala, and Honduras) was bred to create maize (Fukunaga et al. 2005). Together, teosinte and maize compose the genus *Zea*, which has four species: (1) *Z. luxurians* (Durieu and Ascherson) Bird, an annual teosinte from Central America; (2) *Z. diploperennis* Iltis, Doebley and Guzman, a diploid perennial teosinte from Jalisco, Mexico; (3) *Z. perennis* (Hitchc.) Reeves and Mangelsdorf, a tetraploid perennial teosinte from Jalisco, Mexico; and (4) *Z. mays*, a polytypic annual species that includes four subspecies: (1) ssp. *mays* (maize); (2) ssp. *mexicana* (Schrader) Iltis, a large-spikeleted teosinte adapted to the drier high elevations (~1600–2700 m) of northern and central Mexico; (3) ssp.



a. Yield of wheat, rice and maize across the world in hectogram per hectare (hg/ha) (Source: FAOSTATA 2013)



b. Area (Million hectare) and production (Million Tonnes) of wheat, rice and maize across the world for last ten years (FAOSTAT 2013) (Source: FAOSTATA 2013)

Figure 7. Yield and area of production of wheat, rice and maize across the world for last ten years (FAOSTAT 2013) (Source: FAOSTATA 2013)

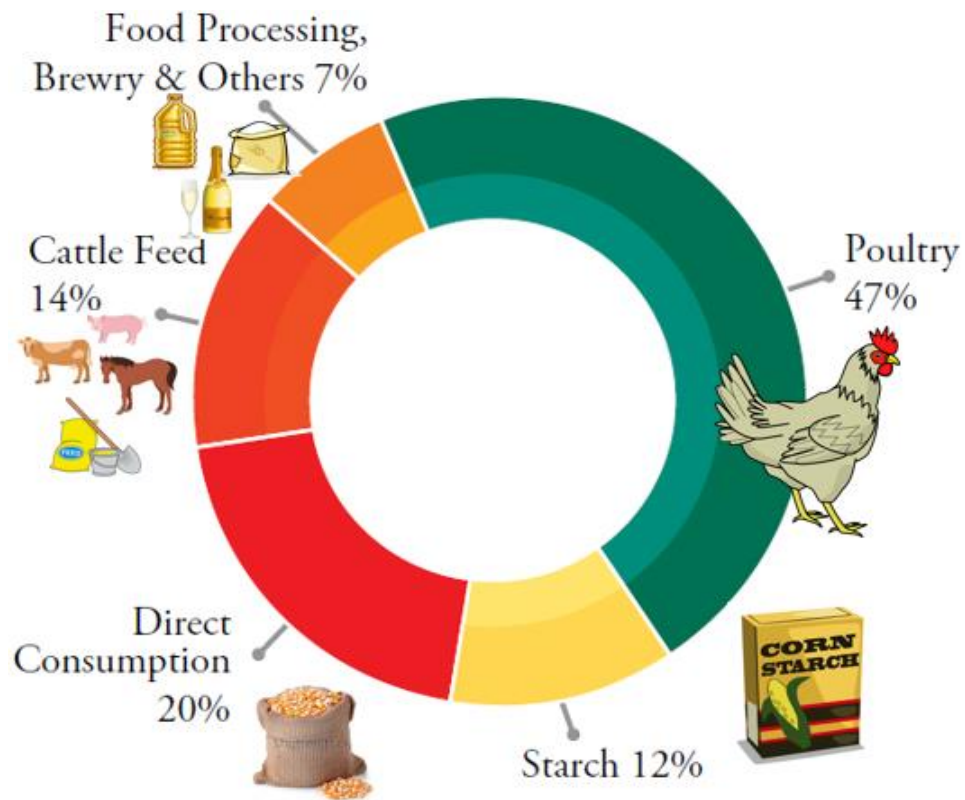


Figure 8. Percentage share of different product of maize
(Source: Indian Maize Summit 2014)

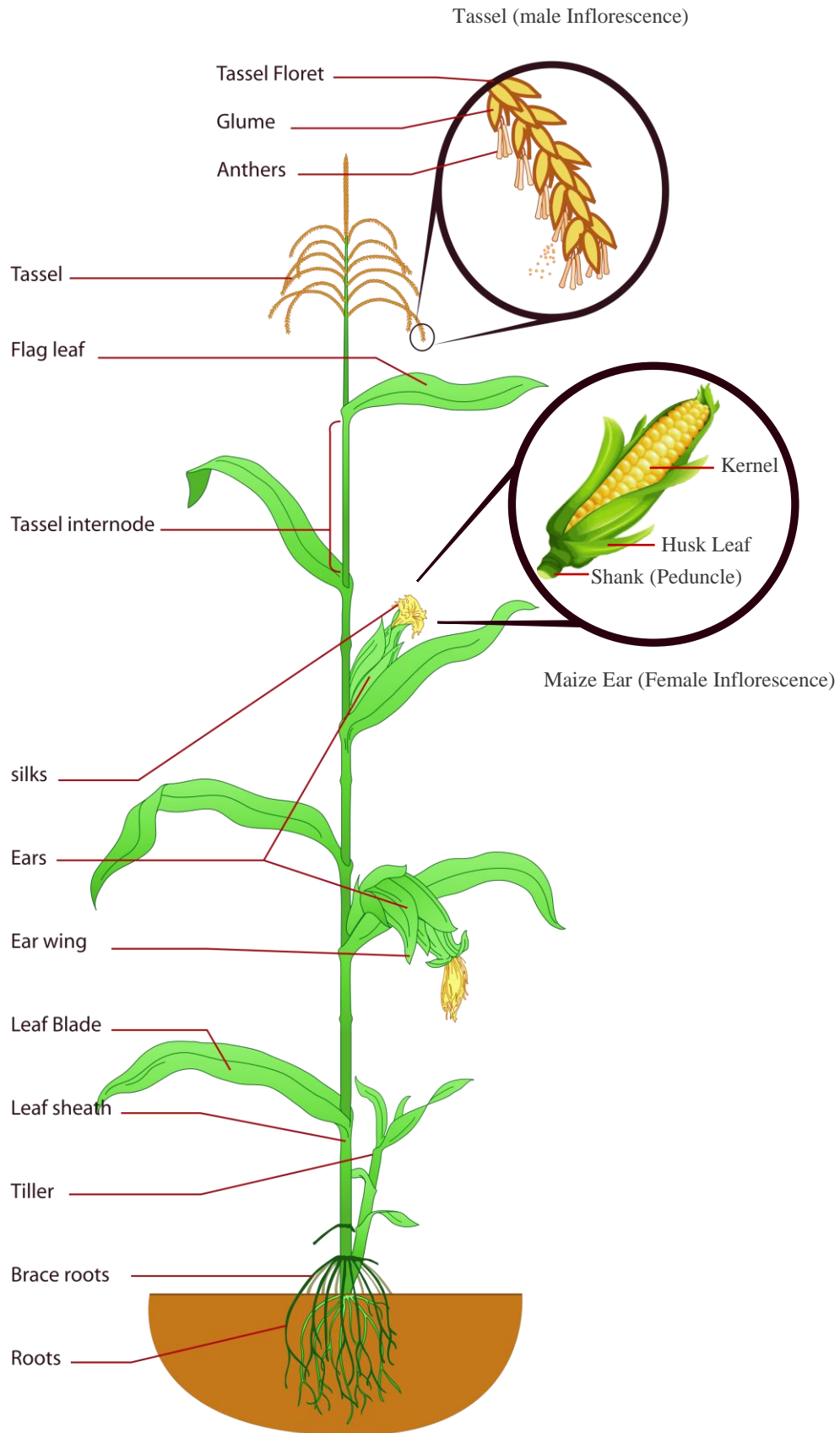


Figure 9. Morphology of Maize Plant
(Source: www.wikicommons.com)

parviglumis Iltis and Doebley, a small-spikeleted teosinte adapted to the moister middle elevation (~400–1800 m) of southwestern Mexico; and (4) ssp. *huehuetenangensis* (Iltis and Doebley) Doebley, an annual teosinte found only in the province of Huehuetenango in western Guatemala (Fukunaga et al. 2005). The four species of *Z. mays* have been placed into two sections: section *Zea*, which contains only *Z. mays* and section *Luxuriantes*, which is composed of the other three species (Fukunaga et al. 2005). The importance of crop can be surmised by the fact that about 60 % of the daily diet of the ancient American civilization known as Maya consisted of maize (Staller 2010). Till now, Americans have developed more than two hundred varieties of maize.

In India maize was introduced in 16th century by the Portuguese, following the discovery of trade-routes by Vasco da Gama in 1498 (Watt 1893). Traditionally, most of the maize was used as feed but in modern time except direct consumption it has several other means of uses such as cattle feed, poultry and for industrial purposes. In India, maize contributes nearly 9 % of the total food basket and more than Rs. 100 billion is added to the agricultural gross domestic product (GDP). In India, consumption has also increased at a CAGR of 3.6 % over the last five years (India Maize Summit 2014). In India, maize is grown throughout the year in a wide range of environments, extending from extreme semi-arid to sub-humid and humid regions but predominantly it is a Kharif crop with 85 % of the area cultivated in this season. The crop is also very popular in the low and mid-hill areas of the western and northeastern regions. The predominant maize growing states contribute more than 80 % of the total maize production are: Andhra Pradesh (20.9 %), Karnataka (16.5 %), Rajasthan (9.9 %), Maharashtra (9.1 %), Bihar (8.9 %), Uttar Pradesh (6.1 %), Madhya Pradesh (5.7 %), Himachal Pradesh (4.4 %) (**Figure 10**). Apart from these states maize is also grown in Jammu and Kashmir and North-Eastern states.

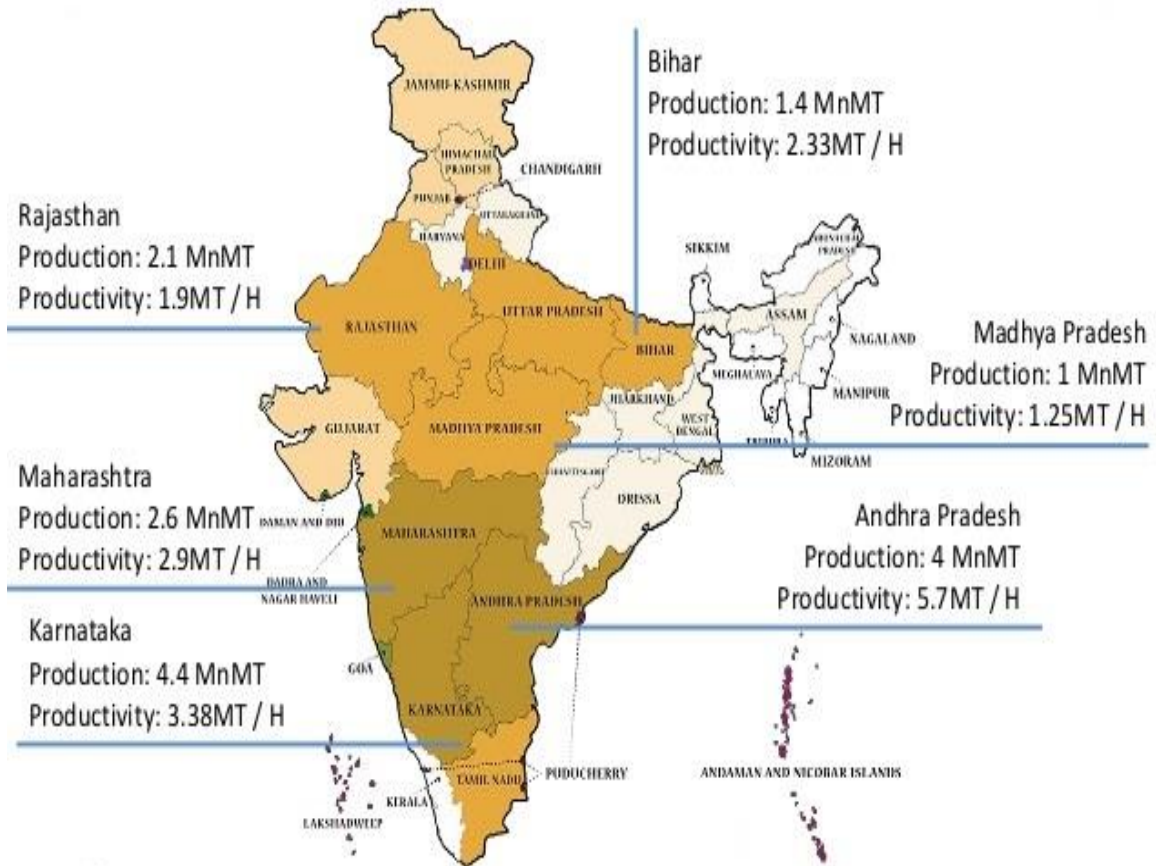


Figure 10. State wise production of maize plant (Source: Indian Maize Summit 2014)

2.1. Maize productivity: Issues related to heavy use of mineral fertilizers and pesticides

Cereal crops are the primary source of food, fodder, fibers and other useful products to sustain life. But in the current scenario where rising human population created more demand for cereal crops, increasing crop productivity is a big challenge for agriculture systems (FAO STAT 2013). In this situation for maintaining the productivity two practices widely adopted in agriculture system are 1) excessive use of chemicals in the form of fertilizers and 2) pesticide application for protection from diseases. Albeit use of mineral fertilizers had greatly improved crop productivity, but their indiscriminate use has also proven harmful to the environment (Byrnes 1990). N and phosphorus (P) are the two most important nutrients limiting maize yields in various parts of the world (Miao et al. 2007). Maize requires high demand of minerals (N, P, K) at different growth stages (Onwueme and Sinha 1991). It has been found that inadequate availability of minerals during the first two to six weeks after planting can result in reduced yield potentials (Jones 1985). However, excessive use of mineral fertilizers for growing maize is vulnerable. It has been found other during application in the field, most of the fertilizers are lost by leaching, erosion, gaseous emissions or other means (Peoples et al. 1995) and cause environmental instability (Miao et al. 2007). According to Ladha et al. (2016), only half of the mineral fertilizer is available for near-term crop production. Studies also indicate that mineral fertilizers applied to various cropping systems can cause both positive and adverse effects on soil organic carbon (SOC) balances and further evaluation of these mineral fertilizers on SOC dynamics for better assessment and agricultural sustainability is required (NAAB 2015). The other side effects of using mineral fertilization are emission of pollutants in the atmosphere, soil, and water (Mueller et al. 2013; Jones 2014). Despite several drawbacks, the global per capita rates of mineral fertilizer consumption per year have increased (Smil 2001). Which indicates that for increasing productivity in agricultural systems mineral fertilizers still dominate. But ecological

perspective more sound approaches such as the use of biofertilizers and biopesticide is required.

For more than a century, different methods used for controlling phytopathogens have been proposed with varying degrees of success. However, among them, chemical control based on synthetic products has been the mainstay of crop protection. Since their immediate effectiveness and ease of application in agriculture, synthetic pesticides very soon became popular as effective means to control a plethora of pests. However, the dark side of their uninhibited use also resulted in the form of destruction of ecosystems and damage to human health (Aktar 2009). Ground water pollution, loss of soil fertility and destruction of biodiversity are attributed to the use of pesticides (Gibbs et al. 2009). According to Pimentel et al. (1996), pesticides use has increased since 1950 at the rate of 2.5 million tons annually, yet crop loss from pests has remained relatively constant. This directly indicates that the indiscriminate use of pesticides is only causing harm. An earlier estimation of World Health Organization (WHO) also stated that 3 million pesticide poisonings occur annually, causing 220,000 deaths (WHO 1992). Pesticides affecting the aquatic ecosystems cause imbalance in the aquatic food web (Bingham 2007). Animals (including humans) are being poisoned by bioaccumulation of pesticides (Agrawal et al. 2010). For the protection of maize against seed-borne fungal diseases, its seed is often pre-treated with a fungicide such as captan, etridiazole, or thiram. Yang et al. (2011) found that these and other similar fungicide are hazardous to other non-target beneficial microorganisms. One more demerit associated with the use of these fungicides is also the development of resistance amongst phytopathogens (Murphy 2007).

2.2. Ear and charcoal rot caused by *F. moniliforme* and *M. phaseolina*: A threat to maize productivity

Since the inception of agriculture practices, crops at various stages of development have been exploited by different phytopathogens. A closer look on plant diseases tells us that there has been a great history of crop losses due to phytopathogens and various outbreaks were reported where phytopathogens remained the main culprits for severe crop loss. For example, epidemics of potato blight known as the great Irish famines (1739 and then 1845-1849) caused the death of 750,000 people and emigration of two million to the United States (Gribben 1999). According to an estimate, 10-20 % of global agricultural production is being affected by phytopathogens and deprives 800 million people of getting adequate food (Strange and Scott 2005). More recently the Food and Agriculture Organization (FAO) estimates that pests and diseases are responsible for about 25% of crop loss (FAO 2015). Developing countries are more vulnerable to loss in the productivity because agriculture plays a lead role in economic growth of the nation (Dubey et al. 2010). Each year diseases caused by fungi, bacteria, plant-parasitic nematodes and viruses result in losses in corn production. Amongst them more than 72 fungal diseases collectively caused rot, blight, mildew and sopt diseases in corn (<http://www.apsnet.org/publications/commonnames/Pages/CornorMaize.aspx>). All parts of the plant may be affected at various stages of development but the infection of ears, stalks and roots directly affect the yield.

Charcoal root rot is an economically important disease caused by the fungus *M. phaseolina* (Tassi) Goidanich and reported in North and South America, Asia and Africa and some parts of Europe. The fungus has a wide host range and estimated to infect about 500 plant species in more than 100 families globally (Srivastava et al. 2001). Under the favorable conditions, bean, sorghum, cotton, soybean and corn are most susceptible to fungal infections

(Mihail 1992). In the absence of reliable classification system, taxonomy of *M. phaseolina* remained unclear. The genus *Macrophomina* was first established by Petrak (1923). However, the pycnidial state of the fungus was originally named *Macrophoma phaseolina* by Tassi (1901) and *Macrophoma phaseoli* by Maublanc (1905). Currently *M. phaseolina* (Tassi.) Goid is applied to both the microsclerotial and pycnidial anamorphs. However the microsclerotial phase is the one predominantly observed worldwide (Dhingra and Sinclair 1977). Many different synonyms have been given to the fungus such as *M. phaseoli* (Maubl.) Ashby, *Macrophoma conchoci* Swada, *M. cajani* Syd. and Butl., *Sclerotium bataticola* Taub., and *Rhizoctonia bataticola* (Taub.) (Butler 1925). However, sub-species of *M. phaseolina* were classified by differences in microsclerotia size, cultural characteristics, chlorate sensitivity, pycnidia formation and pathogenicity (Mihail and Taylor 1995; Suriachandraselvan and Seetharaman 2000). Attempts have also been made to develop faster and unambiguous molecular methods for differentiation of *M. phaseolina* (Jana et al. 2003). As the disease-resistant cultivars for *Macrophomina* are rare and the fungus shows physiological specialization for survival in extreme soil condition, its eradication is very tough. The fungus is reported to be soil, seed and stubble borne. The fungus may survive 2 or more years in dry soil as microsclerotia (Songa and Hillocks 1996) but not more than 7–8 weeks in wet soils and mycelium cannot survive more than seven days (Sinclair 1982). The fungus produces tuber or cushion shaped 1-8 mm diameter black sclerotia which serve as primary means of inoculum (Kaisar and Das 1988). Disease initiate when the root of host plants come in contact of sclerotia living in seed, soil and plant remains (Reuveni et al. 1983). The disease development is favored at optimum temperatures (30-35°C). Disease severity also increases at high temperatures and prolonged drought conditions (Tesso et al. 2005). Similar to other cereal crops, no cultivars with complete resistance to *M. phaseolina* in maize are known, and fungicides are not potent enough to protect the crop (Khan 2007).

The fungus, *Fusarium moniliforme* J. Sheldon (= *F. verticillioides* (Saccardo) Nirenberg, teleomorph = *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura) has been associated to cause ear, stalk, and root rot of corn (Sheldon 1904; Manns and Adams 1921; Sherbakoff 1922). The fungus was originally described by Sheldon (Nelson 1992) in Nebraska in 1904 when he isolated it from ears of corn showing a pink mold, and described as follows: "Sporodochium, subeffuse, salmon-pink; sporophores, simple or branched, usually opposite; microconidia, continuous, oblong ovoid, moniliform, 6-10 μ long; macroconidia, falcate, acute, for the most part three-septate, 25-40 μ long". However, Sherbakoff (1922) set up the section *Moniliform* as follows: "Macroconidia type intermediate between *Roseum* and *Elegans*, thin walls mostly three-septate; microconidia also in chains, chlamydospores none, color of substratum from none to violet." One major economic importance of *F. moniliforme* in maize is the *in-situ* production of mycotoxins that contaminate the stored grain. There are at least 28 different forms of fumonisins, mycotoxins designated as A-series, B-series, C-series, and P-series documented from corn or corn-based feeds and foods (<http://www.apsnet.org/edcenter/intropp/topics/mycotoxins/pages/Fumonisin.aspx>) Amongst all, Fumonisin B1 is the major fumonisin discovered in 1988 (Gelderblom et al. 1988). Toxicological studies have shown that fumonisins cause leukoencephalomalacia (LEM) in horses (Kellerman et al. 1990), pulmonary edema syndrome (PES) in pigs (Colvin and Harrison 1992), liver cancer in rats (Gelderblom et al. 1991) and has been statistically associated with an increased risk of esophageal cancer (EC) in humans consuming contaminated corn (Rheeder et al. 1992). *F. moniliforme* make its entry in corn ears at the silking stage (Warren 1978). Even when ears are covered by husks and free from bird or worm damage they may be contaminated (Koehler 1942). Apart from widespread infection of shelled corn, several other routes in which *F. moniliforme* may infest or infect corn kernels

are documented (Kaiser 1980). Studies also indicate that combination of local and systemic infection may also occur (Christensen and Wilcoxson 1966) or fungi may invade vascular tissues leading from the main stalk to the ear (Kingsland and Wernham 1962). Maize is grown either as continuous monoculture or in short rotations with one or two other crops, fields retaining maize or plant debris in or on the soil are the primary source of inoculum (Smith and White 1988). *Fusarium* spp. may survive on maize residue either as mycelium or other survival structures i.e. microconidia, macroconidia and perithecia (Smith and White 1988). Microconidia are typically the more numerous and more easily wind-dispersed than insects and play the active role in inoculum dispersion (Dowd 1998). Besides this evidence also indicates that heat and drought stress is associated with elevated levels of *F. moniliforme* colonization in corn and high fumonisin accumulation (Bacon and Williamson 1992).

2.3. Plant growth promoting rhizobacteria: ecofriendly solution

The soil is a living system with a vast array of microbes and animals interacting with each other. In the soil, there is an area surrounding to plant roots which influences microbial interactions to a maximum level and it is known as “rhizosphere” (Kennedy 2005). The term first coined by Hiltner (1904) and can be regarded as the basis of understanding all possible biological interactions occurring in the soil. The rhizosphere is the origin place of rhizobacteria that aggressively colonize plant roots (Antoun and Kloepper 2001). The presence of rhizobacteria in the rhizosphere can have a neutral, detrimental or beneficial effect on plant growth. The presence of neutral rhizobacteria in the rhizosphere probably has no effect on plant growth. However, a variety of bacteria in the soils can act as plant pathogens, reducing plant growth, productivity, and fitness that make suppressive soil. The beneficial effect of rhizobacteria on plant growth were first reported by Kloepper and Schroth (1978) while conducting their experiments on radishes. Later these rhizobacteria were named as PGPR (Kloepper and Schroth 1981). In the rhizosphere, PGPR utilize compounds and

materials released from plant roots and in relation to this exert a beneficial effect by improving growth and vigor. Due to both plant growth promotion and protection against soil-borne diseases, they are also termed as plant health-promoting bacteria. PGPR include representatives from very diverse bacterial taxa (Vessey 2003; Lucy et al. 2004) and a number of bacterial genera such as *Azospirillum*, *Alcaligenes*, *Arthobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacteria*, *Pseudomonas*, *Rhizobium* and *Serratia*, etc. are reported to be PGPR (Tilak et al. 2005; Egambrediyeva 2005; Kaewchai et al. 2009; Kumar et al. 2012; Reddy 2014; Arora 2015, Arora et al. 2016; Goswami et al. 2016). According to Jeyarajan and Nakkeeran (2000), an ideal PGPR shows high rhizosphere competence and competitive saprophytic ability, enhance plant growth, have the capability of mass multiplication, shows tolerance to desiccation, heat, oxidizing agents and Ultra Violet (UV) radiations. The concept of utilizing PGPR in sustainable agriculture is now widely accepted (Reddy 2014). Farmers have also started to exploit them in the form of bioinoculants. Use of nitrogen-fixers, phosphate solubilizers and biocontrol activity showing PGPR in the form of bioinoculants also limits the need for costly fertilizers and chemical pesticides in agroecosystems.

The mechanisms for plant growth improvement by PGPR have been extensively reviewed by various authors (Glick 2012; Ahemad and Kibert 2014; Kundan 2015). PGPR mediate overall plant growth promotion by both direct and indirect mechanism (**Figure 11**). Direct mechanism involve improvement of mineral acquisition, production, and secretion of plant growth regulators. However indirect mechanisms are due to suppression plant from phytopathogens and activities related to plant tolerance to biotic and abiotic stress (Glick 1995; Glick et al. 1999; Gupta et al. 2000; Beneduzi et al. 2012).

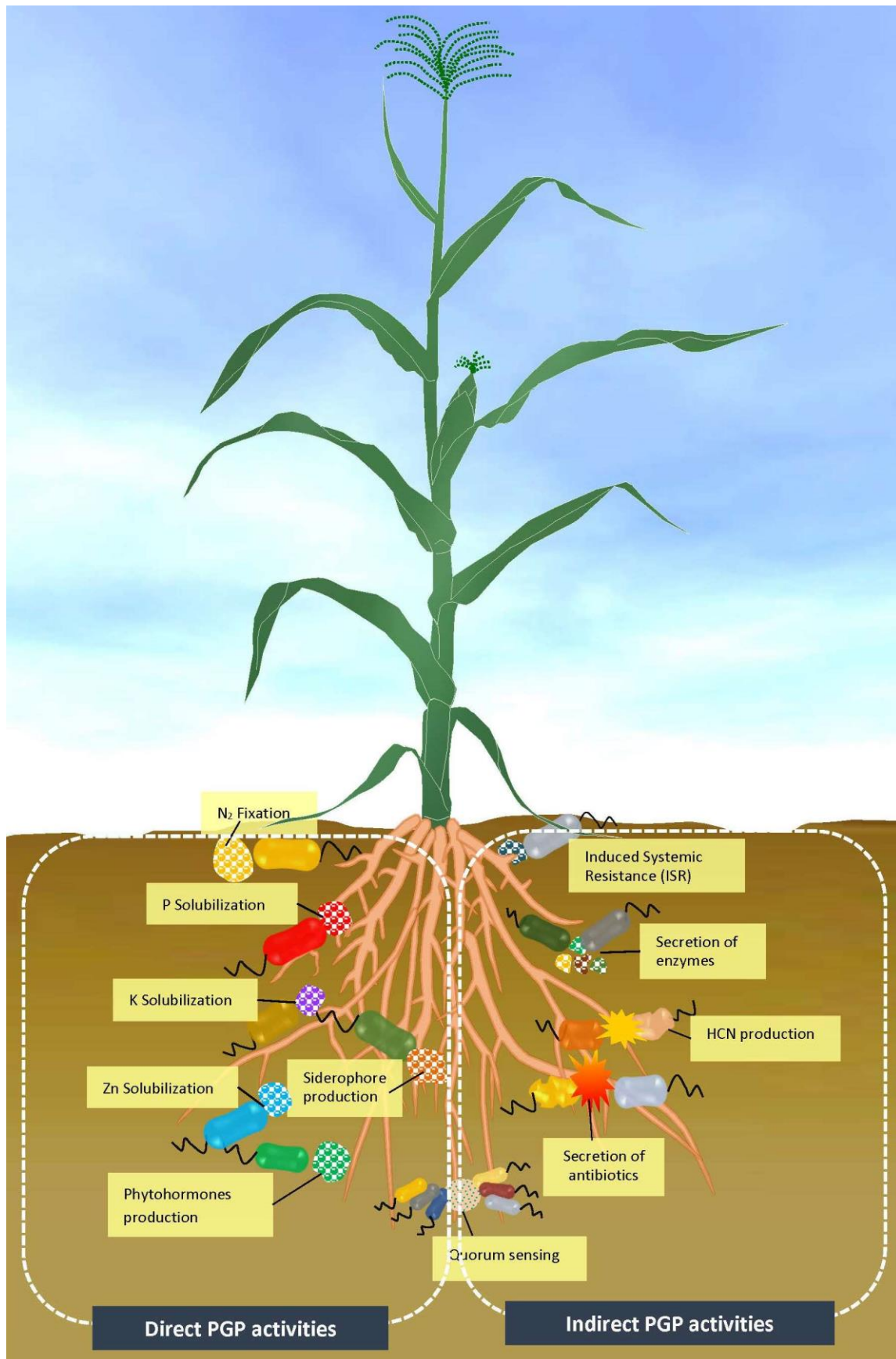


Figure 11. Plant growth promoting activities of PGPR in rhizosphere
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2.3.1. Direct mechanisms

Much of the work indicates that PGPR can easily facilitate uptake of P, K, N, Fe and Zn to plants (Maheshwari et al. 2012). The solubilization and mineralization of P is carried out by phosphate solubilizing bacteria (PSB) by secretion of low molecular weight organic acids such as gluconic and citric acid (Rodriguez et al. 2006). In the presence of acids, soil acidification occurs that results in the release of soluble phosphates (Kpombrekou and Tabatabai 1994; Rodríguez and Fraga 1999; Alikhani 2006). Other mechanisms that are too important are the release of H⁺ (Illmer and Schinner 1992), the production of chelating substances (Sivasakthi et al. 2013) and inorganic acids (Sharma et al. 2013). Some studies also confirmed the indirect participation of exopolysaccharides (EPS) synthesized by PSB in the solubilization of tricalcium phosphates (Yi et al. 2008). Secretion of phytase by various rhizospheric microbes have been demonstrated to be useful in solubilization of insoluble form of organic phosphate (phytate) and increases its availability to plants (Singh et al. 2014). PSB of the genus *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Rhodococcus*, *Serratia*, *Gordonia*, *Phyllobacterium*, *Delftia*, *Azotobacter*, *Xanthomonas*, *Chryseobacterium*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Xanthobacter*, and *Rhizobium* are being marketed as inoculants in various countries (Mishra and Arora 2016). Similar to PSB potassium solubilizing bacteria (KSB) are also able to solubilize rock K mineral powder, such as micas, illite, and orthoclases through production and excretion of organic acids (Friedrich et al. 1991, Ullman et al. 1996). Bacteria of the genus *Pseudomonas*, *Burkholderia*, *Acidithiobacillus*, *Bacillus*, and *Paenibacillus* are effective K solubilizers (Lian et al. 2002, Sheng and He 2006; Liu et al. 2012). Co-inoculation of PSB and KSB have been also found to be useful in increasing P and K availability in plants by using rock materials as a source of P and K (Han et al. 2006). PGPR having the capability of biological nitrogen fixation (BNF) are considered as the best alternatives to N fertilizers at least for legume crops (Gothwal et al. 2009). In agroecosystems

rhizobia and *Azospirillum* spp., are also employed as bacterial inoculants for providing plants N need (Souza 2015; Fukami et al. 2016). The atmospheric dinitrogen (N_2) is fixed exclusively by N_2 -fixing (diazotrophic) bacteria having the enzyme nitrogenase (Dean and Jacobson 1992). In Fe-starved conditions rhizospheric microbes produce a low molecular weight compound known as siderophore which play important role in plant health management (Ahmed and Holmstrom 2014). These molecules show an extraordinarily high affinity for Fe^{+3} (K_a ranging from 10^{23} to 10^{52}). Many important metabolic reactions of the plants are Zn-dependent (Brown et al. 1995). Recently the use of PGPR for Zn uptake, particularly in Zn-deficient soils, has been suggested as a sustainable way to fulfill plant needs (Vivas et al. 2006; Saravanan et al. 2007; Wang et al. 2013; Sunithakumari et al. 2016). PGPR produce organic acid such as acetic, citric, lactic, propionic, glycolic, oxalic, gluconic acid that sequester cations and acidify the microenvironment near the root (Cunnigham and Kuiack 1992; Alexander, 1997). Plants take up Zn as divalent cation (Zn^{2++}). The release of organic acids is thought to be a major mechanism of Zn solubilization.

PGPR can enhance plant growth and development by the production of different phytohormones like auxins, gibberellins (GAs), cytokinins, ethylene and abscisic acid (ABA) (Baca and Elmerich 2007). Amongst naturally occurring auxins, indole-3-acetic acid (IAA) is often found to be produced by various PGPR (Spaepen and Vanderleyden 2011). IAA produced by PGPR take part in the proliferation of lateral and adventitious root that results in improved mineral and nutrient uptake by plants (Steenhoudt and Vanderleyden 2000). Tryptophan is the main IAA precursor in five of the six pathways discovered for auxin biosynthesis in bacteria and classified according to their intermediate as indole-3-acetamide, indole-3-pyruvate, tryptamine, tryptophan side-chain oxidase, indole-3-acetonitrile, and tryptophan-independent, and they have been extensively reviewed (Patten and Glick 1996; Spaepen et al. 2007). GAs are complex diterpene-derived compounds sharing a common

tetracyclic 6-5-6-5 fused hydrocarbon ring (gibberellane) skeletal structure. The first GA gibberellin A (GA₃), was originally isolated as the bioactive component of *Gibberella fujikuroi* (*Fusarium moniliforme*) that causes foolish rice seedling disease (Yabuta 1938). Whereas amongst bacteria gibberellins-like substances were first described in *Azospirillum brasilense* and *Rhizobium* (Tien et al. 1979; Williams and Sicardi de Mallorca 1982). Amongst PGPR legume-associated *Bradyrhizobium japonicum* produces same gibberellin (GA₃) as *G. fujikuroi* (Morone 2008). Now studies have confirmed that various genres such as *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Agrobacterium*, *Clostridium*, *Rhizobium*, *Burkholderia*, and *Xanthomonas* produce GAs (Mitter et al. 2002; Tsakelova et al. 2006; Kang et al. 2009).

Seed inoculation with cytokinin-producing bacteria stimulated shoot growth and reduced root/shoot ratio in droughted affected plants (Arkhipova et al. 2005; Arkhipova et al. 2007). Cytokinins have been detected in the culture medium of several bacteria (Arkhipova et al., 2005; Karadeniz et al., 2006; Ali et al., 2009). In a study, *Rhizobium* enhanced cytokinin production in plants by regulating the expression of signaling pathway and trigger cortical cells to divide in plants (Oldroyd, 2007). Hussain and Hasnain (2009) also found that Cytokinin production by some PGPR enhanced cell division in cucumber cotyledons. Ethylene hormone produced by some PGPR also acts as a stress hormone when plants are exposed to salinity, drought, waterlogging, heavy metals or pathogens (Babaloa 2010).

ABA is necessary for regulation of several events during late seed development and is crucial for the response to environmental stresses such as desiccation, salt and cold (Finkelstein 2013). Recently Porcel et al. (2014) showed the involvement of plant endogenous ABA produced by *Bacillus megaterium* in PGP activity in tomato plants. It has also been noticed that during stressed conditions when concentration of ethylene rises PGPR

possessing the enzyme, ACC deaminase, facilitate plant growth and development by decreasing ethylene levels (Nadeem et al. 2007 and Zahir et al., 2008).

2.3.2. Indirect mechanisms

Biocontrol of phytopathogens is one of the remarkable properties of PGPR. The fundamental mechanisms responsible for biocontrol activity of PGPR are their ability to compete or colonize for space or nutrients, production of antibiotics and enzymes, and induction of systemic resistance against the pathogens (Raaijmakers et al. 2009). However, it is notable that more than one mechanisms may be found in a single strain and act simultaneously (Mavrodi et al. 2012). For example, some PGPR may produce non volatile and volatile metabolites or secrete of cell wall degrading enzymes and siderophores for biocontrol of phytopathogens (Enebak et al. 1998; Kloepper 1993; Weller 1988). ISR or systemic acquired resistance (SAR) involves activation of chemical and physical defenses of the plant host by an inducer including PGPR (Kloepper et al. 1992). PGPR-mediated ISR may be an alternative to the use of chemical inducers or pathogens for inducing SAR.

There are many PGPR which act as biocontrol agents and produces many biocontrol metabolites against phytopathogens (Glick 2012). Amongst them, fluorescent pseudomonads and bacillus are very prominent. Antibiotics, such as polymyxin, circulin and colistin, produced by a majority of *Bacillus* spp. are active against Gram-positive and Gram-negative bacteria, as well as many pathogenic fungi (Maksimov et al., 2011). The major biocontrol metabolites produced by fluorescent pseudomonads are one of the most efficient in the control of plant pathogens: HCN, phenazines, pyrrolnitrin, 2,4- di acetyl phloro glucinol (DAPG), pyoluteorin, and cyclic lipopolypeptides (CLPs) such as viscosinamide and tensin (Fernando et al., 2006; Bhattacharyya and Jha 2012).

There are many PGPR such as *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas* and *Rhizobium* known to produce volatile organic compounds (VOCs) such as HCN that can

suppress the growth of other microorganisms (Siddiqui et al. 2006; Devi et al. 2007; Rezzonico et al. 2007; Ahmad et al., 2008). HCN inhibits many metalloenzymes of respiratory chain including copper (Cu) containing cytochrome C oxidases. In the presence of glycine and HCN synthetase enzyme (associated within the plasma membrane of certain rhizobacteria) HCN is formed (Blumer and Haas 2000). Other volatile compounds which suppress many soil-borne pathogens are antifungal volatiles such as benzothiazole, cyclohexanol, n-decanal, dimethyl trisulfide, 2-ethyl 1-hexanol, and nonanal, produced by fluorescent pseudomonas (Fernando et al. 2004). These volatile compounds completely inhibit the growth of mycelium, germination of ascospores and the survival of sclerotia and thus preventing the disease occurrence. Ryu et al. (2003) reported some strains of *Bacillus* and *Enterobacter* release a blend of volatile components, particularly, 2, 3 -butanediol and acetoin showing biocontrol activity against many phytopathogens.

The production of catabolic enzymes by PGPR is also a way of biocontrol. There are several studies which indicates that enzymes such as proteases, β -1,3-glucanase and chitinases secreted by PGPR contribute in suppression of soilborne plant pathogens (Saraf et al. 2014). Studies also indicates that enzymes causes obvious abnormality of the mycelial growth, which can be attributed to the effect of cell wall- degrading enzymes such as chitinases, produced by rhizobacteria (Zhao et al. 2014).

The phenazines are non-volatile nitrogen-containing colored aromatic secondary metabolites produced by many bacterial species and show broad antibiotic activity against bacteria and fungi (Chin-A-Woeng 2003). Over many different phenazine derivatives are produced by *Pseudomonas*. Amongst them, phenazine-1,6-dicarboxylic acid (PDC) and phenazine-1-carboxylic acid (PCA). PDC act as “core” phenazines (Blankenfeldt and Parsons (2014). Pyocyanin (5-N-methyl-1-hydroxyphenazine) is the most studied phenazine which is blue when oxidized. The proposed modes of action of phenazines include disruption

of energy-dependent membrane associated metabolic processes and DNA intercalation (Picker and Fridovich 1984; Perrin et al. 2000). However, in last few years, the formation of reactive oxygen species (ROS) and oxidative stress by phenazines is also reported mechanism (Laursen and Nielsen 2004; Mavrodi et al. 2006).

2,4-DAPG is a non-nitrogen containing compound and is one of just three phloroglucinols that have been isolated from fluorescent pseudomonads (Shanahan 1992). DAPG can induce plant resistance against pathogens (Showkat et al. 2012). Strains of *P. fluorescens* that produce DAPG are responsible for the natural suppression of take-all diseases of wheat (*Gaeumannomyces graminis* var. *tritici*), known as take-all decline (Raaijmakers et al. 1998; Weller et al. 2002; Weller et al. 2007). DAPG produced by *P. fluorescens*, has also shown broad-spectrum antibiotic activity against viruses, bacteria, fungi, and also known to cause induced systematic resistance in plants (Morrissey et al. 2004).

Pyoluteorin is an aromatic chlorinated polyketide resorcinol ring linked to bichlorinated pyrrole moiety, which was first isolated from *P. aeruginosa* and has bactericidal, herbicidal and fungicidal properties (Takeda 1958). Later, Howell and Stipanovic (1980) reported that pyoluteorin produced by *Pseudomonas fluorescens* Pf-5 suppressed *Pythium*. It was also found that plant host appears to affect pyoluteorin's contribution to biological control (Maurhofer et al. 1994).

Pyrrolnitrin is chlorinated phenylpyrrol antibiotic that was first isolated from *Burkholderia pyrrocinia* (Arima et al. 1964) and was later found in other genera, such as *Pseudomonas*, *Enterobacter*, *Myxococcus* and *Serratia* (Kalbe et al., 1996; Hammer et al., 1999). It has been described as an inhibitor of fungal respiratory chain (Tripathi and Gottlieb 1969). The antibiotic pyrrolnitrin produced by *Pseudomonas* and *Burkholderia* species has shown activity against several economically important pathogens like *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahlia* and *Sclerotinia sclerotiorum* (Ligon et al. 2000).

CLPs are thought to have, antimicrobial (Takesako et al. 1993; Gerard et al., 1997; Vollenbroich *et al.*, 1997) and biosurfactant properties (Rosenberg and Ron, 1999). Production of different kinds of CLP is common among fluorescent Pseudomonads (Nielsen *et al.*, 2002). All CLPs have either 9 or 11 amino acids in the peptide ring with a C10 fatty acid at one of the amino acids (Nielsen et al., 2002). Viscosinainide is a cyclic lipopeptide produced by *P. fluorescens* (Nielsen et al. 1999) and shows prominent antifungal and biosurfactant properties (Nielsen et al. 2000; Thrane et al. 2000; Nielsen et al. 2002).

PGPR also suppress plant diseases by inducing resistance mechanisms known as ISR (Van Loon et al. 1998) which resembles pathogen-induced SAR (Van Loon et al. 1998). ISR and SAR act through different signaling pathways (Beneduzi et al. 2012). Induction of SAR involves salicylic acid (SA) production and ISR involves jasmonic acid (JA) and ethylene (ET) signaling pathways (Van Loon et al. 1998). Even exogenous application of these signaling molecules is found to induce resistance (Ryals et al. 1996). However, protection mediated by ISR is significantly less than that obtained by SAR (Van Loon, 2000) but together ISR and SAR provide a better protection than each of them alone, indicating that they can act additively in inducing resistance to pathogens (Van Wees et al. 2000).

It has also been noticed that in some cases siderophores which have major role in plant iron nutrition may also play an important role in the biocontrol of some soil-borne plant diseases (Loper and Buer 1991). Because siderophores sequester the limited supply of Fe^{+3} present in the rhizosphere, they limit its availability to pathogens and ultimately suppress their growth (Schroth et al. 1984; Paulitz and Loper 1991; Arora et al. 2001). Siderophores produced by various fluorescent pseudomonads in iron limiting conditions is reported to have wider applicability in agroecosystems (Ahmed and Holmström 2014). Pyoverdines are the prevalent type of siderophore which carry both catechol and hydroxamate groups responsible for iron chelation (Leong 1986). Some other siderophores

produced by *Pseudomonas* spp. are pyochelin (Buysens et al. 1996), enantio-pyochelin (Youard et al. 2007), pseudomonine (Mercado- Blanco et al. 2001; Loper et al. 2012), achromobactin (Loper et al. 2012), pseudobactin (Meziane et al. 2005), pseudomonine (Mercado-Blanco et al. 2001) and pyocheline (Audenaert et al. 2002), They may act through competition for iron and thus limiting their availability for pathogens (Bakker et al. 1986; Loper and Buyer 1991).

In last few decades role of PGPR in combating abiotic stresses has gain momentum (Dimkpa et al. 2009; Lim and Kim 2013; Tewari and Arora 2015). The detailed knowledge of the physiological processes involved in enhancing tolerance towards abiotic stresses such as drought and flood has provided new dimensions to enhance crop productivity under harsh environmental conditions (Yang 2009; Tewari and Arora 2015; Tewari and Arora 2016). Studies have concluded that it would be preferable to use such PGPR for providing protection against such stresses. PGPR help plants to tolerate abiotic stresses by various mechanisms (Yang 2009). Amongst them, the production of osmoprotectors (K⁺, glutamate, trehalose, proline, glycine and polysaccharates), stress-induced production of phytohormones (IAA and GAs) and IST are of importance (Yuwono et al. 2005; Saleem et al. 2007; Sziderics et al. 2007; Barriuso et al. 2008). Under stress condition, some PGPR also produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ethylene precursor in plants) (Saleem et al. 2007). By reducing the level of ethylene, the plant becomes more resistant to stress conditions in the environment (Glick 2005).

2.4. Fluorescent pseudomonads

PGPR belonging to the fluorescent group have enormous value and their potential in biological control of plant diseases has gained momentum (Walsh et al. 2001). They are globally being commercialized in the form of bioinoculants for the management of several plant diseases and considered to be very effective (Singh et al. 2016). In the fluorescent

pseudomonads group, most of the research has been carried out on the species *Pseudomonas fluorescens* due to its immense potential as biocontrol agents against a vast array of pathogenic fungi (Weller 2007). Apart from this other notable species are *P. aeruginosa*, *P. putida*, and *P. syringae*. One remarkable feature of all fluorescent pseudomonads is the production of the fluorescent pigment. Saprophytic fluorescent Pseudomonads are typically found in agricultural field soil and involved in several interactions with plants (Kloepper and Schroth 1981). The genus belongs to the class gamma proteobacteria and the family *Pseudomonadaceae* (Order: *Pseudomonadales*) which contains 191 validly described species (Euzéby 1997). The etymology of the name was not earlier provided and first appeared in the 7th edition of Bergey's Manual as Greek pseudo "false" and Monas "a single unit," which can mean false unit. Subsequently, the term "monad" was used in the early history of microbiology to denote single-celled organisms (Palleroni 2010). However, the typed species name "*Pseudomonas aeruginosa*" was given by Walter Migula (1900) by realizing the fact that this strain can be distinguished by its capacity to synthesize pigments (aerugo is the Latin word for verdigris, the blue-greenish copper rust). Members of the genus display the following defining characteristics: rod-shaped, Gram-negative, one or more polar flagella for providing motility, non-spore forming, and positive catalase test, aerobic respiration (some strains also have anaerobic respiration with nitrate as the terminal electron acceptor and/or arginine fermentation), many species accumulate poly-hydroxybutyrate (PHB) as carbon reserve material, show metabolic versatility, and a high genomic G+C content (59–68%). The multi-generic nature of *Pseudomonas* was confirmed by studies of Palleroni and collaborators (1973). By measuring rRNA: DNA hybridization, they subdivided the genus into five distantly related so-called rRNA groups (rRNA groups I to V). However, phylogenetic distribution of the pseudomonads is now done by combining data from 16S rRNA sequence analysis, rRNA-DNA hybridization and polyphasic taxonomic studies (including DNA: DNA

hybridization). The genus *Pseudomonas* is now restricted to the rRNA group I and includes 36 genuine *Pseudomonas* species which show similarity in genomic and phenotypic characteristics to the type species *Pseudomonas aeruginosa* (Kerstens et al. 1996). The majority of the other species have been reclassified as genera *Burkholderia*, *Ralstonia*, *Brevundimonas*, *Sphingomonas*, *Xanthomonas*, *Stenotrophomonas* and as members of the family *Comamonadaceae* comprising the genera *Acidovorax*, *Comamonas* and *Hydrogenophaga* (Kerstens et al. 1996). In iron deficiency conditions, *Pseudomonas* species do not accumulate PHB, and produce a yellow-green pigment that fluoresces under UV radiation. This substance was first observed by Gessard (1892) and named bacterial fluorescein and more recently pyoverdine. That's the reason of referring them as fluorescent pseudomonads. In Bergey's Manual of Systematic Bacteriology (Palleroni 1984), species included in this group are *P. aeruginosa*, *P. aureofaciens* (now *P. chlororaphis*), *P. cichorii*, *P. fluorescens*, *P. putida*, *P. syringae* and *P. viridiflava*.

As earlier discussed, similar to other potent biocontrol PGPR, fluorescent pseudomonads also utilize secondary metabolites production as the primary mechanism of biocontrol and experimental evidence clearly supports that in the biocontrol of phytopathogens HCN, phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin and CLPs are involved (**Figure 12**).

According to Schippers et al. (1990) depending on environmental factors and plant species, certain strains of rhizosphere *Pseudomonas* spp. produce HCN which may inhibit establishment or inhibit development of plant disease. In a study Lantaigne et al. (2012) showed simultaneous DAPG/HCN production by *Pseudomonas* sp. LBUM300 with great potential for controlling bacterial canker of tomato. Recently, Reetha et al. (2014) also found that HCN produced by *Pseudomonas* CF1 and CF5 inhibited growth of *M. phaseolina*. It has

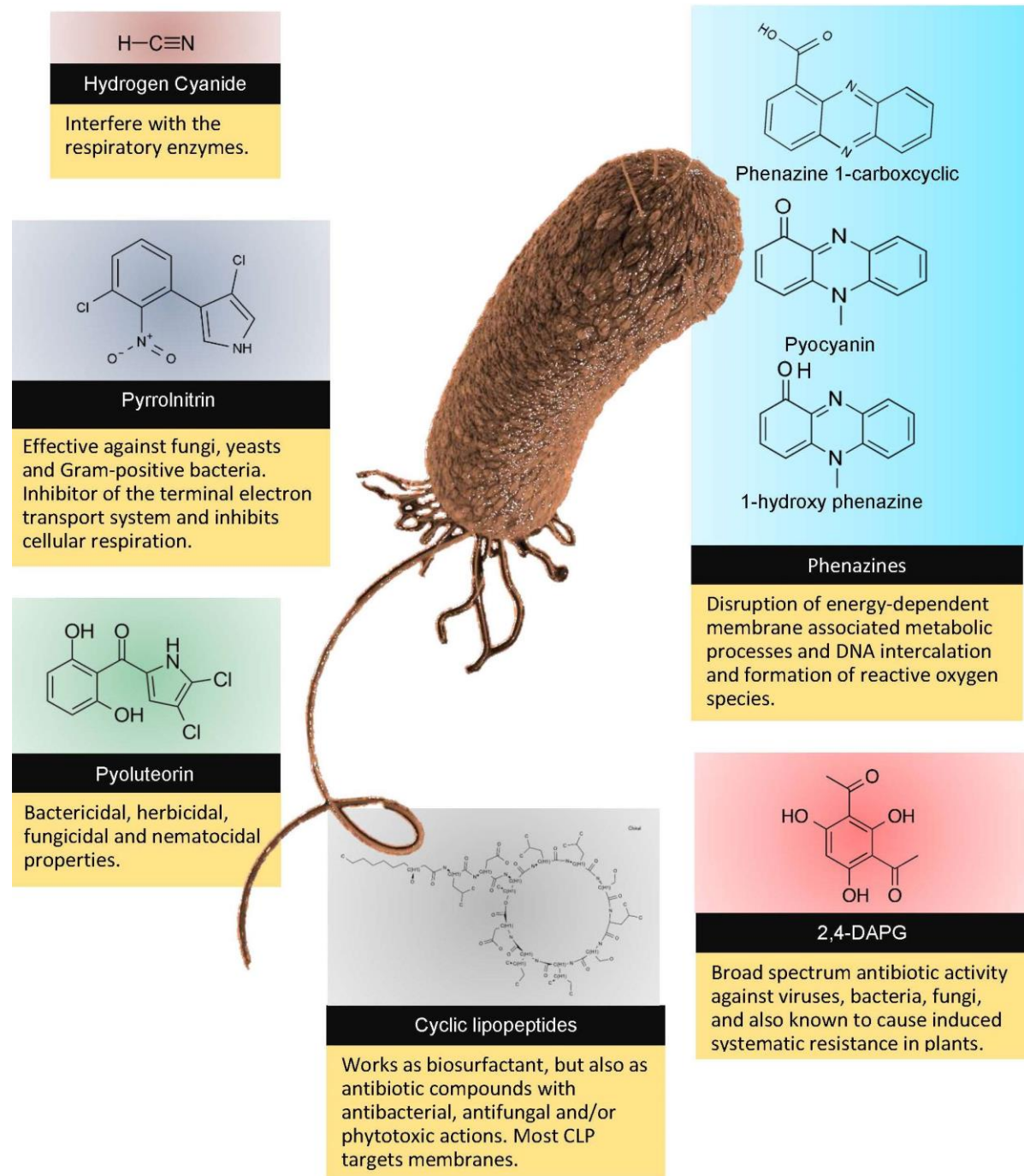


Figure 12. Biocontrol secondary metabolites of fluorescent pseudomonads
(Image is copyright of Mishra and Arora)

been also noticed that along with other biocontrol antifungal metabolites, HCN inhibits growth of many seed borne fungi (Pal et al. 2001).

There are evidences where production of catabolic enzymes by fluorescent pseudomonads were reported to suppress growth of phytopathogen fungi (Ueda et al. 1995). Amongst them β 1,3 glucanase has ability to control a wide array of phytopathogenic fungi (Hamid et al. 2013; Jose et al. 2014). In a study Arora et al. (2007) found that fluorescent pseudomonads producing chitinase and beta-1,3-glucanase enzymes showed in vitro inhibition of *Phytophthora capsici* and *Rhizoctonia solani*.

PDC, PCA and pyocyanin phenazines are very found very effective in treatment of fungal diseases (Chincholkar and Thomashow 2014). Pyrrolnitrin produced by *P. cepacia* was found to inhibit the growth of the fungus *Colletotrichum truncatum* and also exhibited antifungal activity against the potato dry rot fungus *Fusarium sambucinum*, which was the first report of pyrrolnitrin activity against *Fusarium* (Burkhead et al. 1994). In a study, Cartwright et al. (1995) found that pyrrolnitrin and phenazine production by *Pseudomonas cepacia*, strain 5.5B suppressed *Rhizoctonia solani* causing stem rot of poinsettia.

The antibiotic DAPG is exclusively produced by pseudomonads of worldwide origin, and its biosynthetic locus is conserved in pseudomonads obtained from diverse geographic locations (Keel et al. 1996; Raaijmakers et al. 1997). Different DAPG producing strains of *P. fluorescence* such as Pf5, CHA0, F113, Q2-87, Q8r1-96 have been used to suppress the black root rot of tobacco, root rot of tomato, Pythium damping-off of cucumber and sugar beet, cyst nematode and soft rot of potato and take-all of wheat (Ayyadurai 2006). Various studies indicate that DAPG reduces Fusarium-wilt caused by *F. oxysporum* f.sp. *cepae*, (Kawamoto and Lorbeer 1976), *F. oxysporum* f.sp. *vasinfectum*, (Howell and Stipanovic 1980) and *F. graminearum* (Ventura et al. 1997).

In last few years, role of certain CLPs isolated from biocontrol strains of *Pseudomonas* spp. were reported to have very good antibiotics and antifungal activities. In a study Nielsen (2002) found that CLPs produced by fluorescent pseudomonads also inhibited mycelial growth of soil borne fungus *Rhizoctonia solani* and *P. ultimum*.

In this way, all these attributes make fluorescent pseudomonads as good candidates in preparation of bioinoculants having wider used in agriculture and can compete with chemical based formulations (Arora et al. 2016). However, studies demonstrating effect of these metabolites on management of phytopathogen of maize are rare and effective product is not available in the market.

2.5. Bioformulation for maize

Improvement and increment of maize productivity in an eco-friendly manner is on priority for maize growers across the globe (Laditi et al. 2011). This can be done by application of PGPR on maize field in the form bioformulation. The use of microbial inoculum in PGP and eradication of phytopathogens has been reported when “Nitragin” bioformulation containing *Rhizobium* sp., was first registered in U.S. (Nobbe and Hiltner 1896). Slowly other bioformulations also developed. Interest in using pseudomonads based bioformulations for biocontrol of phytopathogens was initiated in the 1970s and 1980s (Burr et al. 1998). Earlier reports confirmed that antagonistic fluorescent pseudomonads containing bioformulations were able to suppress take-all disease of wheat and barley (Smiley 1979; Weller 1988). Later it was found that fluorescent pseudomonads containing biformulations could be a good choice to protect the plant from various phytopathogens. Literature indicates that *P. fluorescens* is also being used as bioinoculant for controlling soil and seed borne diseases (Gupta et al. 2002; Khan and Gangopadhyay 2008). Although there have been several incidences where fluorescent pseudomonads based bioformulations provide cure to phytopathogenic fungi but very few or negligible reports are available on biocontrol of ear

and charcoal rot in maize. In a greenhouse trial, Govindappa et al. (2011) found that fluorescent pseudomonads isolates reduced the root rot disease severity. Meena and Marimuthu (2012) also observed the effect of *P. fluorescens* Pf formulation on plant height, leaf area index, root length, nodules per plant and dry matter production. Recently in a study Vanitha and Ramjegathes (2014) revealed biocontrol potential of *P. fluorescens* Pf1 strain against *M. phaseolina* causing root rot disease in coleus. Fluorescent pseudomonads based bioformulation could be the best alternatives to chemicals in preventing ear rot diseases in maize. In a study Nayaka et al. (2009) found that biocontrol of ear rot can be done by employing *P. fluorescens* containing bioformulation. Recently the use of metabolite in bioformulations is also proven successful. For example, rhamnolipid biosurfactant produced by fluorescent *Pseudomonas* was found very effective to treat stalk and ear rot disease of maize (Borah et al. 2016). Tewari and Arora (2015) reported bioformulation containing exopolysaccharides from *P. aeruginosa* PF23 showing effective biocontrol of *M. phaseolina*. Even in saline field conditions, application of bioformulation was promising in enhancing the growth of sunflower and suppressing charcoal rot disease.

Bioformulation having the capability to control seed borne diseases and simultaneous growth promotion should be preferred. But for developing such bioformulations vigorous research is a required and there should implementation of suitable technologies for transferring laboratory knowledge to field level. Carrier involved in development of bioformulation preparations also play a very important role. Selection of suitable carrier may enhance the effectiveness of formulation product (Stephens and Rask 2000; Hungri'a et al. 2005). Although mass production of microbial active ingredients can be achieved through either liquid or solid carrier however use of solid carriers is preferable (Mishra and Arora 2016). Various inert materials such as clay, peat, vermiculite, alginate, and polyacrylamide beads, diatomaceous earth, talc, vermiculite and polymers have been used as solid carriers

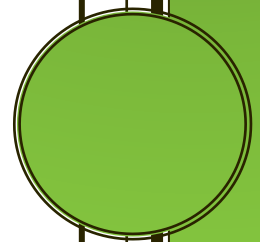
(Digat 1989; Kraus and Loper 1992). Selection of a suitable solid carrier and its efficiency depends on various other factors (Berg 2009). Talc which is chemically referred to as magnesium silicate [$Mg_3Si_4O_{10}(OH)_2$] and easily available as an inert powder is used as a carrier for bioformulation at a large scale. It has very low moisture equilibrium, relative hydrophobicity and chemical inertness, reduced moisture absorption and prevents the formation of hydrate bridges that enable longer storage periods (Jambhulkar and Sharma (2014). The addition of certain gums and sticker also increase the viability of bacterial population in formulations. In earlier studies, Hofte et al. (1991) and De Freitas and Germida (1992) reported that addition of carboxymethylcellulose (CMC) as additive improved the maize seedling emergence. Some other workers also noticed that talc, when used as a solid carrier, gives good results (Chakraborty et al. 2009; Tewari and Arora 2014). In a study Goswami et al. (2013) found that *Pseudomonas* containing talc bioformulation showed significant growth promotion of Chickpea and green gram. In a similar study Jambhulkar and Sharma (2014) showed that *P. fluorescens* RRb-11 containing talc based bioformulation gave best results when applied as seed treatment, seedling root dip and soil application. Jorjani et al. (2012) confirmed that amongst different organic and inorganic carrier used, talc, peat and bentonite based formulation were more effective in controlling sugar beet mortality disease in comparison to Carboxin-thiram fungicides.

Although, this emerging field of using bioformulation instead of synthetic chemicals in agriculture is very effective yet wider applicability has not been noticed. Using fluorescent pseudomonads in the form of active components in the bioformulation is also a matter of intensive research. Amongst all, biocontrol metabolites produced by fluorescent pseudomonads, only few have been tested to control charcoal and ear rot diseases in maize and further research is required in this direction. As it is evident from the discussion above that the damage caused by these two diseases has a vast impact on crop yield and current

eradication measure are not so effective, more robust biological means are urgently required. Besides this, a bioformulation which could be useful in both enhancing maize growth and also to reduce disease severity has not been developed as yet.

Newer approaches also strengthen our knowledge of understanding intricate phenomenon involved in the interaction of fluorescent pseudomonads with their host plant and target pathogen. Apart from this identification of those fluorescent pseudomonads strains which can produce more than one type of biocontrol metabolites against fungal diseases and their use in bioformulations will definitely be more effective. Development of bioformulation according to requirements can solve the problem associated with particular crop such as maize.

MATERIALS AND METHODS



3. MATERIALS AND METHODS

3.1. Collection of soil samples

In total 42 fluorescent pseudomonads (**Table 1**) were isolated from rhizosphere of different plants growing in different regions of Lucknow (26.45°N 81.00°E), Sitapur (27.58°N 80.66°E), Hardoi (27.25°N 80.15°E), Safedabad (26.91°N 81.14°E) and Ballia (25.75°N 84.14°E) region of Uttar Pradesh, India (**Figure 13**). Samples were carried in sterilized polyethylene bags and stored at 4°C for further use.

3.2. Isolation of fluorescent pseudomonads (King et al. 1954)

Soil sample were serially diluted to 10^{-7} dilution and plated in triplicate on sterilized King's B (KB) medium (see appendix). Petri plates were then incubated at 28°C for 2 d. Colonies that fluoresced under UV light were selected and further purified on same medium. All the isolates were also stored on KB agar slants at 4°C for further use (**Figure 14**).

3.3. Phenotypic and biochemical characterization of isolates

3.3.1. Phenotypic characterization

Nutrient agar (see appendix) plates were streaked and incubated at 30°C for 72 h. The plates were observed for development of shape, size, color, morphology and texture according to Bergey's Manual of Systematic Bacteriology (Garrity 2005).

3.3.1.1. Gram Staining (Gram 1884)

Gram staining was done according to Gram (1884). For this log phase culture of the isolated strains were smeared on slides and fixed. The smear was flooded by crystal violet for 45 seconds then rinsed with distilled water. A few drop of gram's iodine was put on the smear for 45 s, followed by washing with 95% ethyl alcohol drop by drop and then washed with distilled water. Finally, the smear was flooded by Safranin for 1 minute and again washed with distilled water. The slide was then observed under oil immersion lens of the microscope for determining color and shape.

3.3.1.2. Motility test

Motility medium (see appendix) was prepared and by using an inoculating needle, stabbed the inoculation in screw cap tubes and incubated the test tube at 27°C for 24 h. After incubation, tubes were examined for the motility.

3.3.2. Biochemical characterization

3.3.2.1. Oxidase test (Kovacs 1956)

Oxidase discs (saturated with 1% aqueous tetra methyl-para-phenylene-diamine solution) were kept on nutrient agar plates and on to them 24 h. old cultures were spotted. A dark purple color, appearing within 10 s, indicates a positive oxidase test.

3.3.2.2. Catalase activity (Graham and Parker 1964)

Colonies on nutrient agar were flooded with 3% hydrogen peroxide (H₂O₂) and observed for liberation of effervescence of oxygen around the bacterial colonies.

3.3.2.3. Citrate utilization (Beishir 1991)

Simmons citrate agar media (See appendix) was prepared and bacterial strains were streaked at the bottom of slant and incubated for 24 h. at 28°C. After incubation change in the color of the media was observed.

3.3.2.4. Indole test

Tryptophan broth (see appendix) was prepared and poured in test tube. Isolated strains were inoculated in the test tubes and incubated for 72 h. at 28°C. To check the indole production Kovac's reagent was added and observed for color change.

3.3.2.5. Methyl-Red and Voges-Proskauer (MR-VP) test (Mac Faddin and Jean 2000)

MR-VP Broth (see appendix) was prepared and 5ml of broth was poured in 2 sets of test tubes then inoculated with 24 h old culture and incubated for 48 h at 28°C. After incubation, 5 drops of methyl red were added in 1 set of test tube and 12 drops V-P reagent I (naphthol solution) and 2-3 drops of V-P reagent II (40% potassium hydroxide) in another set. The appearance of red color in MR test and crimson red to ruby color indicates positive result.

3.3.2.6. Oxidative-fermentative (OF) test (Hugh and Leifson 1953)

OF medium (see appendix) was sterilized and added in two set of test tubes, a filter sterilized solution of 10% solution of glucose was aseptically added to the medium to a final concentration of 1%. Both tubes were Inoculated with isolates using a straight wire by stabbing “half way to the bottom” of the tube. One tube of each pair was covered with 1 cm layer of sterile mineral oil or liquid paraffin. All tubes were incubated at 35°C for 48 h and observed for color change.

3.3.2.7. Levan Production (Lelliot et al. 1966; Dye and Kemp 1977)

For this Petri dishes containing KB Agar were prepared; after solidification of medium half of the medium was cut with the help of sterile slide and removed in the vacant half of the petri dishes KB medium amended with 5% sucrose (w/v) were streaked with a loopful of a pure culture of the bacterial strain. Strains that were positive for levan production showed large, mucoid, domed colonies after seven d at 25°C.

3.3.2.8. Gelatinase Test (Chapman 1952)

Nutrient gelatin (see appendix) was prepared and transferred to test tubes. Test tubes were stab inoculated with bacterial culture and incubated 25± 2°C for one week. Secreted gelatinase will liquefy the medium is considered positive.

3.3.2.9. Urease test (Christensen 1946)

Christensen's urea medium (see Appendix) was prepared and after sterilization in test tubes (5.0ml/tube) medium and 0.25 ml of sterile urea solution was added. Slant of the urea containing medium were inoculated with isolates and incubated at 28± °C. The medium turned from colorless to red in the presence of urease.

3.3.2.10. Starch hydrolysis test (Lennette et al. 1985)

Starch Agar media (See appendix) was prepared and isolates were spot inoculated on the plate and incubated for 48 h at 27°C. After incubation plates were flooded with solution of Gram's iodine solution for 4-5 m. After removing the iodine solution, plates were observed for the colorless zone around colonies.

3.3.2.11. Casein hydrolysis (Blackburn 1968)

Skim milk agar media (see appendix) was prepared and isolates were spot inoculated on the plate and incubated for 24 h at 28°C for analysis of zone of clearance around the colonies.

3.3.2.12. Lipase test (Samad et al. 1989)

Lipase agar medium (see appendix) was made and spot inoculated with bacterial isolates. After 24 h of incubation at 28°C colonies with lipolytic activity were observed by seeing opacity due to calcium salt crystals formed by lipolysis of fatty acid.

3.3.2.13. Arginine dihydrolase test (Taylor and Whitby 1964)

Each isolate was tested for arginine dihydrolase production. The medium containing L-arginine was dispensed in 2-ml amounts in test tubes (control tube without L-arginine) and autoclaved at 121°C for 20 m and stored at 4°C. Both tubes were inoculated by stabbing the media one half way to the bottom. They were then covered with a 5-mm layer of sterile liquid paraffin, tightly capped, and incubated at 28°C for 7 d. The tubes were observed on d 1, 2, 5, and 7 for positive or negative results. A positive test for the presence of arginine dihydrolase was indicated by a deep pink to red color, whereas a negative test was demonstrated by an absence of color change in the medium or a yellow color.

3.3.2.14. Growth at 4°C and 42°C (Oberhofer 1979)

Tests for growth at 4°C and 42°C were performed with Trypticase soy agar (see appendix) slants placed in an incubator adjusted to 4°C and 42°C and the growth was observed after 24 and 48 h.

3.3.2.15. Pyocyanin production (Price-Whelan et al. 2006).

The 48-h old culture of isolates in KB was centrifuged at 20,000×g and supernatant filtered through 0.22µm pore. The filtered supernatant was measured spectrophotometrically at 690 nm and pyocyanin concentration were calculated using extinction coefficient for pyocyanin ($\epsilon=4,310 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 7).

3.4. Plant growth promoting and biocontrol activities

3.4.1. Phosphate solubilization (Pikovskaya 1948)

Pikovskaya's agar medium (see appendix) was prepared and log phase culture (0.5µl) of all isolates were spot inoculated on this medium and incubated at 28°C for 4-5 d. After incubation colonies showing halo zones around were used for determination of solubilization index (SI) by using the following formula (Edi-Premono et al. 1996).

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

3.4.2. Zinc solubilization

The modified Pikovskaya medium (Pikovskaya 1948) specified by Saravanan et al. (2007) containing (g/L) glucose: 10.0; (NH₄)₂SO₄: 1.0; KCl: 0.2; K₂HPO₄: 0.2; MgSO₄: 0.1; yeast extract powder:0.2; pH: 7.0 and insoluble Zn compound (ZnO) at concentration of 0.1% and agar: 15.0 was autoclaved at 121°C for 20 m. Log phase cultures (0.5 µl) of each strain were spot-inoculated onto the agar plates and plates were incubated at 28°C for 4-5 d. The clearing zone around colony was recorded in the form of zinc solubilization index (ZSI).

3.4.3. IAA activity (Brick et al. 1991)

To check IAA production isolates were incubated for 3 d at 28°C without any shaking in Minimal medium (see Appendix), supplemented with 0.2 g/l L-tryptophan. Fully grown cultures were centrifuged at 8000 rpm for 10 m. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl₃ solution). Development of pink color indicates IAA production. For

quantitative estimation, a standard curve prepared with known concentration in the range of 10–100 mg/ml of IAA and absorbance was taken at 530 nm with the help of spectrophotometer (Evolution 201, Thermo Scientific). The quantity in the culture filtrate was determined and expressed as mg/ml.

3.4.4. Gibberellic acid (GA)

GA was determined by the method of Holbrook et al. (1961) and Sivasakthivelan and Stella (2012). For this 100 ml of KB broth was inoculated with bacterial strains and then incubated in a shaking incubator at 150 rpm at 28°C for seven d. After this 7.5 ml culture was pipetted out to which 1 ml of zinc acetate and 1 ml of potassium ferrocyanide added and centrifuged at 8000 g for 10 m. After that 2.5 ml of supernatant was taken and to which 2.5 ml 30% HCl was added and incubated at 27°C for 75 m. After that its absorbance was taken at 254 nm. The amount of GA was calculated in μ g/ml using the standard curve of GA dissolved in acetone.

3.4.5. Siderophore production

All the fluorescent pseudomonad isolates were screened for their potential to chelate Fe in soil.

For this various siderophore producing tests were carried out:

3.4.5.1. Addition of Fe salts (Payne 1994)

For this K B medium was prepared and autoclaved for 15 m at 15 lbs pressure and 121°C and poured into Petri dishes; once the medium has solidified half of the medium was cut with a sterile glass slide and removed then in remaining portion, K B amended with 50 mg/l FeCl₃ was poured. Petri plates were incubated at 27-30°C for three d and observed for the presence of brown color on medium amended with FeCl₃.

3.4.5.2. Chrome azurol sulphonate assay (Schwyn and Neilands 1987)

It is a universal test for the detection and determination of siderophore. The test was carried out method given by Schwyn and Neilands (1987) by accepting minor modification according to Perez-Miranda et al. (2007). For this CAS agar plates were prepared and spot inoculated

with bacterial strains and observed for development of orange halo against dark blue background around the colonies after 48h of incubation at 28°C.

The quantitative estimation of siderophore was also performed. For this succinate broth (sees appendix) was prepared (Meyer and Abdallah 1978) and inoculated with bacterial strains. This was followed by incubation in a shaking incubator at 28°C and 150 rpm for 5 d. After incubation cultures were centrifuged at 6000 rpm for 15 m to obtain cell free supernatant for further use. The following reagents were also prepared to accomplish the test:

1. 2mM CAS stock solution by adding of 0.121 g CAS in 100 ml water.
 2. 1 mM Fe stock solution by dissolving 1 mMFeCl₃•6H₂O in 10 mM HCl
 3. Piperazine-N, N-bis (ethanesulfonic acid) (PIPES) Buffer by addition of 4.032 gm PIPES in 100 ml of distilled water and maintaining pH 5.6 by addition of 1N NaOH
 4. Hexadecyltrimethylammonium bromide (HDTMA) solution by dissolving 0.0219 g HDTMA in 50 ml water.
 5. CAS Assay Solution: 1.5 ml Fe solution is mixed with 7.5 ml CAS stock solution to which HDTMA is added in a reagent bottle and mixed it 36.75ml of PIPES buffer solution is added and the volume is brought up to 100 ml with distilled water.
 6. Shuttle solution: 0.2 M 5-Sulfosalicylic acid solution is prepared and stored in the dark.
- 2 ml of cell free supernatant of each isolate was added to test tube. Then 2ml of CAS assay solution was added and mixed after this 40 µl shuttle solution was also added and mixed. The solution was left for few m and then absorbance taken at 630 nm. The succinate medium was used as blank, and succinate medium plus CAS assay solution plus shuttle solution was used as a reference. Siderophore units are defined as

$$\frac{A_r - A_s}{A_r} \times 100 = \% \text{ Siderophore unit}$$

here, A_r = absorbance of reference at 630 nm (CAS reagent) and A_s = absorbance of sample at 630 nm.

3.4.5.3. Chemical characterization of siderophores

All the isolates which showed a color change in Fe salt test and given positive CAS result were also tested for types of siderophore production by following methods:

3.4.5.3.1. Hydroxymate type (Neilands 1981)

To 1 ml of cell free supernatant of isolates 1.5 ml freshly prepared 2% aqueous solution of $FeCl_3$ was added. Absorbance between 400 to 600 nm was taken and peak between 420-450 nm indicates hydroxymate type.

3.4.5.3.2. Catecholate type (Arnow 1937)

This assay was performed according to Arnow (1937) for quantitative and qualitative estimation of catecholate type of siderophores. The following reagents were prepared:

1. Nitrite-molybdate reagent by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate 100 ml water.
2. 0.5 N HCl
3. 1 N NaOH

To 1 ml of cell free supernatant, reagents were added in the following order and mixing after each: 1 ml HCl, 1 ml nitrite-molybdate (catechols produce a yellow color at this point), and 1 ml NaOH (color should change to red). The color was stable for at least one hr. and the solution had shown an absorption maximum at 510 nm. A standard curve was also prepared by using 2,3 di-hydroxy-benzoic acid for quantitative estimation.

3.4.5.3.3. Carboxylate type (Vogel 1992)

For qualitative determination of carboxylate type siderophore, in 300 μ l 2 N NaOH, 100 μ l phenolphthalein indicator was added which changed color of the solution to pink. After this 100

μ l cell free supernatant was added to it. A colorless solution indicates the presence of carboxylate type of siderophores.

In another qualitative test carboxylate group that belong to siderophore only were assessed according to Shenker et al. (1992). For this 1 ml of cell free supernatant and 1 ml of 250 μ M CuSO₄ and 2ml of acetate buffer were added; the complex copper showed an absorption maximum between 190-280 nm.

3.4.5.3.4. Salicylate type (Nagarajkumar et al. 2004)

This is used for qualitative determination of salicylate type siderophores. For this bacterial strains were grown at 28°C for 48 h on a rotary shaker in 100 ml conical flasks containing 50 ml of the succinate medium. Cells were then centrifuged at 6000 g for 5 m and 4 ml of cell free culture supernatant was acidified with 1 N HCl to pH 2.0 and salicylic acid (SA) was extracted in CHCl₃ (2ml). To the pooled CHCl₃ phases, 4ml of distilled water and 5ml of 2M FeCl₃ were added. The absorbance of the purple Fe-SA complex, developed in the aqueous phase was read at 527 nm. A standard curve was prepared with SA dissolved in succinate medium. The quantity of SA in the culture filtrate was expressed as mg/ml (Meyer et al. 1992).

3.4.6. HCN production (Bakker and Schippers 1987)

HCN induction media (by adding 4.4 g/l glycine in KB medium) was prepared and log phase culture of bacterial strains were spread. A Whatman No. 1 filter paper dipped in 2% sodium carbonate prepared in 0.5% picric acid solution was placed in the lid of Petri plates. The plates were sealed with parafilm and incubated at 28°C for 4 ds in microaerophilic conditions. After incubation color change (yellow to brown) in filter paper was visually assessed against the control.

3.5. *In-vitro* biocontrol activities

3.5.1. Phytopathogenic fungal strains

Phytopathogenic strain of *M. phaseolina* ARIF CC257 was procured from Mycology and Plant Pathology Group, Division of Plant Sciences, Agharkar Research Institute, Pune. However fungal pathogen *F. moniliforme* ITCC No. 2193 was procured from division of Plant Pathology, Indian Type Culture Collection, IARI, New Delhi. Both fungal strains were grown and maintained on Potato Dextrose Agar (PDA) (see appendix) at 28°C and 4°C.

3.5.2. *In-vitro* biocontrol activities against *M. phaseolina* and *F. moniliforme*

All the isolates were tested for their *in vitro* antifungal activity against *M. phaseolina* and *F. moniliforme*. For this (with help of cork-borer) a mycelial disc of 5 mm in diameter of each fungal strain was excised from the edge of an actively growing antagonist on PDA plate and placed at the center of a fresh PDA plate 24 h culture of each bacterial strain was streaked on either side of the fungal disc (equidistant from the periphery). In the control, only mycelial discs were placed. The plates were kept for incubation at 27°C for 5 d. After the incubation period, radial growth of pathogens was measured and the percent inhibition of average radial growth was calculated relative to the control following the equation of Udomsilp et al. (2009) as follows:

$$L = \frac{C - T}{C} \times 100$$

where L is the percentage inhibition of radial mycelial growth, C is radial growth of the pathogen in the control; T is radial growth of the pathogen in the presence of isolated strains.

3.4.6. Hydrolytic enzymatic activity

Those isolates showed best result biocontrol activity against these two fungi were also checked for the production of hydrolytic enzymes: cellulase, chitinase and β -1,3 glucanase. Cellulase was measured according to Hankin and Anagostakis (1977). For this 2% CMC containing plates were prepared and spot inoculated with isolates. Then incubated at 28 °C for 8 d and

observed for clear halo zone formation. Extracellular chitinase was determined by spot inoculation on solid chitin minimal medium. However, β -1,3 glucanase was assayed according to Dunne et al. (1977).

3.6. Molecular characterization

On the basis of plant growth promoting and biocontrol activities, isolate PFJM1 was selected for molecular characterization. For this 16-sRNA sequencing was carried out by Yaaz Xenomics, India (<http://www.yaazxenomics.com>). Following protocol was used:

3.6.1. DNA Extraction

Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit – As per the instructions following procedure was followed.

- An isolated bacterial colony was picked and suspended in 1ml of sterile water in a microfuge tube.
- Centrifuged for 1 m at 10,000–12,000 rpm to remove the supernatant.
- Added 200 μ l of InstaGene matrix to the pellet and incubated at 56°C for 15 m.
- Vortexed at high speed for 10 s and placed the tube in a 100°C in heat block or boiling water bath for 8 m.
- Finally, vortexed the content at high speed for 10 s and Spinned at 10,000–12,000 rpm for 2 m.
- In result, 20 μ l of the supernatant was used per 50 μ l PCR reaction.

3.6.2. PCR Protocol

16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

Primer Details

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

1µL of template DNA was added in 20 µL of PCR reaction solution. 27F/1492R primers was used for bacteria, and then PCR reaction performed with below conditions:

Initial Denaturation 94°C for 2 m and then 35 amplification cycles at 94°C for 45 s, 55°C for 60 s and 72°C for 60 s. Final Extension at 72°C for 10 m. DNA fragments were amplified for about 1,400 bp in the case of bacteria. Positive control (*E. coli* genomic DNA) and a negative control was also included.

3.6.3. Purification of PCR products

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

3.6.4. Sequencing protocol

Single-pass sequencing was performed on each template using 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequencing Primer Details:

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

3.6.5. Bioinformatics protocol

The 16s r RNA sequence was blast using NCBI BLAST similarity search tool. The phylogeny analysis of sequence with the closely related sequence of BLAST results was performed followed by multiple sequence alignment (Edgar 2004).

3.6.6. GenBank accession number

16s rRNA sequence was submitted to NCBI GenBank database for obtaining accession number.

3.7. Production and extraction of crude antifungal metabolite produced by PFJM1

The bacterial strain PFJM1 which showed strong *in vitro* antifungal activity against *M. phaseolina* and *F. moniliforme* was used for the antifungal metabolite production. For this modified method as proposed by Raaijmakers and Weller (2001) was used.

3.7.1. Inoculum preparation and culture conditions for metabolite production

Briefly, pre-inoculum of PFJM1 was prepared in 5 ml of KB broth and allowed to grow at 30°C for 12 h. Batch fermentation was performed in Erlenmeyer conical flasks containing 250 ml of medium (peptone 20 g, glycerol 20 ml, sodium chloride 5 g, potassium nitrate 1 g dissolved in 1000 ml of distilled water, pH 7±0.2). This was followed by inoculation with 1% of pre-inoculum, and incubated at 28±2°C for 5 d on a rotary shaker at 240 rpm. After fermentation culture was centrifuged at 12000 g for 20 m at 4°C to recover the cell-free supernatant

3.7.2. Extraction of metabolites

The procedure given by Zhou et al. (2012) was used. In brief the bacterial cell free supernatant was acidified to pH 2 and extracted by equal volume of chloroform in a separating funnel. Half of the volume of the separating funnel was filled with supernatant, and the remaining half was filled with solvent. The funnel was shaken well for mixing the supernatant and solvent and kept undisturbed in the stand for half an hour. The separated organic layers were then combined, dried over anhydrous Na₂SO₄, and subjected to evaporation to 1 ml with a rotary evaporator (40°C, 250 rpm). The metabolite was re-suspended in 0.5 ml methanol and stored at 20°C for further analysis. Effect of this crude metabolite on growth of *M. phaseolina* and *F. moniliforme* was also assessed by making well at one corner of PDA plates after transfer of actively grown fungi in the center.

3.7.3. Purification of crude extract by thin layer chromatography

The chloroform crude extract was fractionated by thin layer chromatography (TLC) using silica Gel GRM7480 (Himedia, India). The glass plates (20×20 cm; 0.25 thick) were activated at 110°C for 30 m, cooled, and crude extract was spotted. TLC plates were developed with solvent system of methanol: chloroform (1: 9) according to DeSouza and Raaijmakers (2003). The R_f values of the spots were determined under UV light (254 nm) after TLC running.

3.8. Activity of fractionated metabolite extract against *M. phaseolina* and *F. moniliforme*

After TLC, the active fractions obtained were identified for *in-vitro* antifungal activity against *F. moniliforme* and *M. phaseolina*. For this, agar well diffusion method was used. In Petri plates containing 20 ml of PDA, 20 µl of metabolite suspended in methanol was placed in a well at a distance of 1.0 cm from the edge of the plate. With the help of cork-borer a mycelial disc of 5 mm in diameter of each fungal strain was excised from the edge of an actively growing antagonist on PDA plate and placed at the center of a fresh PDA plate. In control, only methanol was suspended in wells. The plates were incubated at 28°C for 5 d.

3.9. Partial characterization of metabolite

In vitro antifungal activity showing fraction of metabolite were further partially characterized by UV-visible spectrophotometry and fourier transmission infra-red (FT-IR) spectrophotometry.

3.9.1. Characterization by UV-Visible Spectrophotometry

The absorbance maximum of the fractioned extracts was determined by scanning its dilute solution in diethyl ether against pure diethyl ether using UV-Visible Spectrophotometer (Evolution 201, Thermo Scientific) in the region of 200 - 1000 nm

3.9.2. Characterization by Fourier Transmission-Infrared (FT-IR) spectrophotometry

The functional group of the compound was characterized by FT-IR Spectrophotometer (Nicolet TM 6700, Thermo Scientific, USA). The spectrum was measured from 4,000 to 500 cm⁻¹.

3.10. Antagonistic interaction of PFJM1 with fungal pathogen examined under scanning electron microscope (SEM)

Structural abnormality, hyphal lysis, and deformity in conidia formation induced by metabolite produced by bacterial strain were examined under SEM (Model Jeol Quanta 250) at various resolutions (2500 to 6000X). With the help of microneedle mycelia from the zone of interaction with bacteria were mounted on stub fixed with carbon adhesive tape. Further fixation at 4 °C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) was done and washed three times (10 m each) in phosphate buffer and again with distilled water. Then serially dehydrated in 70, 80, 90 and 100% ethanol (5 m in each stage) and three changes in 100% ethanol at room temperature. After this stubs were coated with gold and were observed under SEM.

3.11. *In-vivo* pot experiment

Pot experiment was conducted for two consecutive year (2015-16) during the month of July. For this 5 kg sandy loam soil having pH 8.78 and electrical conductivity (EC) 197 $\mu\text{S}/\text{cm}$ (sterilized 3 consecutive d at 15 psi for 30 m) was filled in earthen pots (50 x 34 x 26 cm). Fungal inoculum was prepared on oat grains (*Avena sativa*) so as to get 10^4 fungal propagules g^{-1} of soil. The maize seeds (Survarna NMH589) were surface sterilized with 0.5% NaOCl solution for 1–2 m, rinsed three times in sterilized distilled water and dried under a sterile air stream. Bacterial isolate PFJM1 was cultivated in KB broth and incubated at 150 rpm in shaking incubator at 28 °C for 12–18 h to obtain late exponential growth phase (Meesungnoen et al. 2012). The bacterial cells density was adjusted to 10^8 CFU/ml ($A_{600\text{nm}} = 0.5$). The cell suspension was mixed with 1% CMC solution and coated on seed then allowed to air-dry overnight under aseptic conditions. The seeds coated with 1% CMC slurry without bacteria served as control. The experiment was conducted in following set of treatments taking triplicate of each set: T1- non-bacterized seed control; T2- PFJM1; T3- *M. phaseolina*; T4- *M. phaseolina*+PFJM1; T5- *F. moniliforme*; T6- *F. moniliforme*+PFJM1. In total five seeds were

sown in a pot by selection of healthiest seedlings. One week after seed germination, the seedlings were thinned to three in each pot. Pots were kept in the open with average day and night temperature of 36°C and 28°C respectively. Moisture was maintained by watering the pots as per requirement. Seed germination was tested 10 d after sowing (DAS). Plant growth parameters including root and shoot length, fresh and dry weight of plant, ear fresh and dry weight, number of seeds per plant and 100 grain weight were measure 90 DAS.

3.12. Bioformulation development

3.12.1. Preparation of bacterial suspension

The inoculum for bioformulation was produced by transferring one loopful of the PFJM1 culture in 100 ml KB broth in a 250 ml Erlenmeyer flask followed by incubation at room temperature ($28 \pm 2^\circ\text{C}$) on a shaker at 150 rpm for 48 h. The cell density obtained was 9.5×10^{10} CFU ml⁻¹.

3.12.2. Inorganic carrier

Talcum powder were obtained from HiMedia, Mumbai India. One Kg of the carrier powder with 20% moisture was autoclaved at 121°C for 30 m for three consecutive d with an overnight incubation in between. To adjust neutral pH, 15 g calcium carbonate was added to sterile powder.

3.12.3. Formulation preparation

Broth containing 9.5×10^{10} CFU ml⁻¹ of PFJM1 strain was used for the preparation of formulations. Formulation was prepared according to Vidhyasekaran and Muthamilan (1995). For this 98-ml culture broth, 1 ml glycerol (1 g ml⁻¹) as a carbon source and 1 g CMC g⁻¹ as an adhesive were added. To make 100 g of talc-based formulation, 80 g of sterilized carrier and 20 ml of culture broth with additives were mixed under sterile conditions. Then the product was shade-dried to reduce the moisture content to 18% and packed in UV-sterilized polythene

bags and sealed (**Figure 15**). Finally, talcum powder formulations contained 1.7×10^{10} CFU ml⁻¹ at the time of packaging

3.12.4. Shelf life assessment of bacterial viability

The viable cell count in bioformulation was determined on K B agar plates at the end of 0, 30, 30, 60, 90, 120, 150 and 180 d of incubation using serial dilution method. 1 g of formulation was suspended in 9 ml of sterile saline solution and serially diluted to 10^{-7} . Average cell number was estimated by calculating CFU on KB agar plates.

3.13. *In-vivo* field experiment

The bioformulation of PFJM1 isolate was evaluated in field experiments for two consecutive years (2015-16) during months of July to September and results obtained were represented as mean of two years. One field trial was conducted in greenhouse field of BBA University and second at Itaunja, Sitapur, UP, Lucknow.

3.13.1. Physicochemical analysis of soil (Jackson 1973)

Soil of both the fields were analyzed for pH, EC, available P (kg ha⁻¹), total N (kg ha⁻¹), exchangeable K (kg ha⁻¹) and organic carbon (kg⁻¹). All the analysis was conducted at Crop Protection Division, CSIR-CIMAP, Lucknow.

3.13.2. Field experiment: BBA University greenhouse

The field experiment was conducted at University greenhouse (26.7679° N, 80.9263° E). The field is naturally infested with *M. phaseolina* (10^3 CFU/g soil). The field experiment was set up as a randomized complete block design with three replicates. Each block was of 75 m² (2.5 m x 3.0 m). In each block, there were 64 plants (8 rows x 8 columns) with a distance 0.20 m between each plant and 0.30 m row width. In total, there were 9 such blocks (3 treatments x 3 replicates) with inter distance of 0.30 m. The treatments were as follows: T1 (seed without any treatment), T2 (seed coated with 5g/kg of Thiram fungicide) and T3 (seed coated with PFJM1 bioformulation). The blocks were irrigated as per requirement. Seed germination was recorded

15 d after the germination. The plants were harvested at the end of 90 d to observe the effect on growth parameters: root and shoot length, fresh and dry weight of plant, ear fresh and dry weight, number of seeds per plant and 100 grain weight. Disease incidence was measured by method given by Ullstrup (1949) and Nowell (1997) which involved observation of rotted ear in comparison to healthy ear.

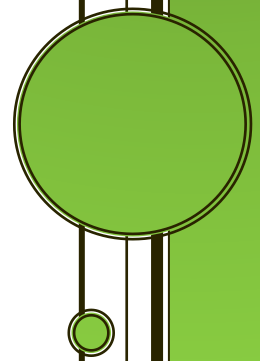
3.13.3. Field experiment: Itaunja

Second field trial was conducted in the month July to October 2016 at Itaunja (27.0808° N, 80.8959° E) U.P., India. This was selected because prevalence of ear rot was observable and soil was found to be infested with ear rot fungi *F. moniliforme* (10^3 CFU/g soil) (**Figure 16**). Experimental scheme was same as in greenhouse field. The plants were harvested 90 DAS to observe the effect on growth parameters, including root and shoot length, fresh and dry weight of plant, ear fresh and dry weight, number of seeds per plant and 100 grain weight. Disease incidence was assessed by observing stalk lodging and premature death of plants.

3.14. Statistical analysis

The data generated during quantitative evaluation of plant growth promotion values were analyzed by means of ANOVA and means were compared by Duncan's Multiplicity Range Test (DMRT) using the SPSS software (Version 21). The significance level for all analysis was $P=0.05$.

RESULTS



4. RESULTS

4.1. Isolation of fluorescent pseudomonads

In total, 42 fluorescent pseudomonads were isolated from different plants. All isolates showed yellowish green pigment when streaked on KB plates (**Figure 17**).

4.2. Phenotypic and biochemical characterization of isolates

All the isolates were Gram-negative, motile and slightly curved rods (**Table 2**). All isolates were oxidase, catalase, arginine dihydrolase, urease positive, indole and MR-VP negative (**Table 3** and **Figure 18**). Isolates were found to utilize citrate and showed the oxidative mode of physiology. Amongst all 91% isolates showed levan production, 95% showed gelatin liquification. Only 5% isolates hydrolyzed starch, 16% showed casein hydrolysis and 7% produced lipase. Only 3 isolates were able to grow at 4°C and none of the isolates showed growth at 41°C. None of the isolates showed pyocyanin production.

4.3. Plant growth promoting and biocontrol activities

4.3.1. Phosphate solubilization

Amongst all, 86% isolates showed phosphate solubilization (**Table 4**) as evident by clear zone. Amongst all PFJM1 showed maximum solubilization of phosphate having PSI of 4.372 (**Figure 19**) Some isolates were moderate solubilizers and their PSI ranged from 2 to 3 (**Figure 20**).

4.3.2. Zinc solubilization

Out of total, 79% isolates solubilized Zn in modified Pikovskaya medium containing ZnO at concentration of 1% (**Table 5**). Amongst all PFJM1 showed maximum solubilization having ZSI of 3.2. (**Figure 21**) Remaining isolates also solubilized Zn in the range of 1 to 2.0 of ZSI (**Figure 22**)

4.3.3. IAA activity

When supplemented with exogenous L-tryptophan, IAA production significantly varied from strain to strain (**Table 6**). Isolate PFJM1 produced maximum amount of IAA as evident from dark pink color (**Figure 23**). Its concentration was 23.109 mg/l. However, other strains were also found to produce IAA as evident by the appearance of light pink color developed by adding Salkowski reagent (**Figure 24**).

4.3.4. Gibberellic acid

All isolates were found to produce GA whose concentration ranged from 0.09 to 4.353 mg/l (**Table 7**). Amongst all PFJM1 was identified as good GA producer and produced 4.353 mg/l. Result also indicated that some isolates which showed IAA production, were also produced GA (**Figure 25, 26**).

4.3.5. Siderophore production

4.3.5.1. Addition of Fe salts

In all 52% isolates produced brown color on medium amended with FeCl₃ (**Figure 27**) and confirms their capability to produce siderophore. These strains were chosen for further siderophore assays.

4.3.5.2. CAS assay

All isolates showing brown color in Fe salt amended plate were also found to give positive CAS assay and showed halo zone in CAS agar plates (**Figure 28**). This was further confirmed by qualitative CAS test where instant decolorization of CAS reagent from blue to orange-red was observed. In CAS test, isolate PFS1 showed maximum (36%) siderophore units. However, isolate PFJM1 showed only 4.5% of siderophores unit.

4.3.5.3. Chemical Characterization of siderophores

Amongst all five isolates were reported to produced more than one type of siderophore. These were PFJM1, PFH1, PFL1, PFL9 and PFSF1. (**Table 8**).

4.3.6. HCN production

Amongst all the isolates, 33 % showed HCN production as evident from dark brown color to light brown color of filter paper. Amongst all PFJM1 was found to produce high amount of HCN (**Figure 29**).

4.3.7. In-vitro biocontrol activities against *M. phaseolina* and *F. moniliforme*

All the isolates were tested for their *in-vitro* antifungal activity against *M. phaseolina* and *F. moniliforme*. Amongst all, only 23% isolates were found to show varying levels of biocontrol activity against both phytopathogenic fungi (**Table 9** and **Figure 30, 31** and **32**). The isolate PFJM1 was identified as a potential antagonist against both fungi. It showed 77% reduction in *M. phaseolina* and 69 % of *F. moniliforme* on dual culture plates.

4.3.7. Hydrolytic enzymatic activity

None of the isolates showed cellulase and β -1,3 glucanases activity. PFJM1 showed minor chitinase activity.

4.4. Molecular characterization

The comparison of 16-sRNA gene sequencing of the isolates with other fluorescent *Pseudomonas* group (taxid:136843) showed 99% similarities which confirmed isolate to be *Pseudomonas fluorescens* JM-1 with the accession number of KT734728.1 (**Figure 33**)

4.5. Production and extraction of crude antifungal metabolite produced by *P. fluorescens* JM-1

Antifungal metabolite was efficiently extracted by chloroform and the crude extract was screened for its activity against *M. phaseolina* and *F. moniliforme*. The crude extract showed inhibition of both the fungi (**Figure 34**).

4.6. Purification of crude extract by thin layer chromatography

TLC was used for the initial separation of the compounds in the crude metabolite extract. The crude metabolite of the strain *P. fluorescens* JM-1 revealed the presence of two major spots with R_f values of 0.74 and 0.78 on TLC plate (**Figure 35**).

4.7. Activity of fractionated metabolite extract against *M. phaseolina* and *F. moniliforme*

The fractions spotted on L-plate was subjected to *in vitro* antagonistic activity, and only one fraction (fraction 2) showed inhibition of *M. phaseolina* and *F. moniliforme* growth (**Figure 36**).

4.8. Characterization by UV-Visible Spectrophotometry

The absorbance maximum of the fractionated extracts was determined by scanning its dilute solution in diethyl ether against pure diethyl ether using UV-Visible Spectrophotometer. UV scan spectrum showed absorbance measured at 270 nm (**Figure 37**).

4.9. Characterization by Fourier Transmission-Infrared (FT-IR) spectrophotometry

The FT-IR spectrum of fractionated metabolite showed characteristic absorption bands from 3300 cm⁻¹ to 600 cm⁻¹ (**Table 10** and **Figure 38**). Functional groups of an average of 40 scans in this frequency range were determined as described by Socrates (2001) and found to be alcohol (OH) (in phenol); R (CH₃) (attached to benzene ring); carbonyl group; C–OH in alcohols, alcohols, ethers and acid esters. These resemble with metabolite DAPG as also reported by Ayyadurai et al. (2006) and Reddy et al. (2007).

4.10. Antagonistic interaction of PFJM1 with fungal pathogen examined under SEM

Structural abnormality, hyphal lysis, and deformity of conidia induced by metabolite produced by *P. fluorescens* JM-1 were observed under SEM. In case of *M. phaseolina* bending of mycelium, fragmentation, absence of sclerotia formation and other structural deformities were observed (**Figure 39**). However, in case of *F. moniliforme* both mycelial and conidial deformities were observed (**Figure 40** and **41**).

4.11. *In-vivo* pot experiment

Results are mentioned in **Table 11** and **Figure 42, 43, 44, 45 and 46**. Seed biopriming with PFJM1 showed significant increase in germination in comparison with non-primed seeds (control). Seed biopriming with PFJM1 brought 27% increment in germination in comparison to control. In case of treatment of PFJM1 in presence of *M. phaseolina* there was 43% enhancement in germination in comparison phytopathogen alone. This was very significant as the soil infested with *M. phaseolina* severely affects germination. In case of *F. moniliforme*+PFJM1 treatment, 22 % increment was also observed in comparison to *negative control*. Amongst all PFJM1 showed best results. PFJM1 brought increment in shoot and root length, plant fresh weight, plant dry weight by 25, 34, 49 and 38% respectively, in comparison to control. Increment in ear fresh weight, dry weight, number of seeds per plant and 100 grain weight was 46, 39, 28 and 43% respectively in comparison to control. The treatment of PFJM1 even in presence of phytopathogens also showed improved growth in comparison to pathogens alone. *M. phaseolina* + PFJM1 treatment was found to increase 6% increment in shoot length, 70.62% root length, 27% plant fresh weight, 64% plant dry weight, 14% ear fresh weight, 33% ear dry weight, 55% number of seed per plant and 56% of 100 grain weight in comparison to control. Treatment of *F. moniliforme*+PFJM1 also significantly increased growth parameters in comparison to pathogen control. It showed 7% increment in shoot length, 19% root length, 13% plant fresh weight, 75% plant dry weight, 31% ear fresh weight, 7% ear dry weight, 28% number of seed per plant and 37% of 100 grain weight in comparison to pathogen control.

4.12. Bioformulation development and field trials

4.12.1. Shelf life assessment of bacterial viability

Talc based bioformulation of PFJM1 supported survival of bacterial population up to 180 days. The **Figure 47** shows decrease in CFU g⁻¹ with time when PFJM1 bioformulation was stored in at 28°C in an incubator. In first 60 days CFU g⁻¹ decreased from 1.7x10¹⁰ to 6.7x10⁹. A

further decrease from 6.7×10^9 to 1.4×10^9 was noticed till 120 d. However, after 180 days the viable CFU g^{-1} was 1.5×10^8 which fulfill the Bureau of Indian Standard (BSI) criterion for number of cells (10^8) required for bioformulation.

4.12.2. *In-vivo* field experiment

The bioformulation of PFJM1 isolate was evaluated in two fields experiments: one at greenhouse field of BBA University and second at Itaunja, Sitapur, UP, Lucknow.

4.12.3. Physicochemical analysis of soil

The soil of both the field was analyzed for pH, EC, available P ($kg\ ha^{-1}$), total N ($kg\ ha^{-1}$), exchangeable K ($kg\ ha^{-1}$) and organic carbon (kg^{-1}). Physicochemical parameters of soil revealed that soil of Itaunja field was more fertile in comparison to BBA University greenhouse soil (**Table 12**) and its pH, EC, available P, total N, exchangeable K and organic C were 7.95, 137.62, 35.68, 125.01, 93.88 and 2.26 respectively. However, pH, EC, available P, total N, exchangeable K and organic C of greenhouse field were 8.52, 116, 17.14, 108.73, 82.8 and 1.72 respectively.

4.12.4. Field experiment: BBA University greenhouse

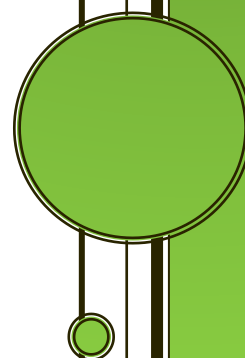
The data of field experiment showed that bioformulation of PFJM1 was very effective in enhancing maize growth in *M. phaseolina* infested soil. (**Table 13** and **Figure 48** and **49**). There was 67% disease reduction was also observed in block treated with PFJM1 bioformulation. The seed coated with PFJM1 bioformulation enhanced germination by 19% in comparison to untreated seed. However, shoot length, root length was increased by 33% and 116%. Similarly plants fresh weight and dry weight were increased by 155%, 111%. In this treatment ear formation was also improved over untreated control and found to increase 87% and 56% in terms of ear fresh and dry weight respectively. Enhancement of seed yield was also higher in PFJM1 treatment over control and here seed per plant and 100 grain weight increased by 43% and 140% respectively. Apart from this seed coated with PFJM1 bioformulation given

better results over seed treated with fungicide thiram. In comparison to fungicide, PFJM1 bioformulation showed increment of 4 % in germination. Whereas as other growth parameters were also significantly increased. In comparison to thiram treatment, increment in shoot length and root length was 30 % and 69%. However, in fresh weight and dry weight increment was 75% and 65 %. Yield in terms of ear fresh weight and dry weight increased by 39 % and 3% and similarly yield in terms of seed per plant and 100 grain weight were recorded to increase by 11 % and 35 % over thiram treatment.

4.12.5. Field experiment: Itaunja

In case of Itaunja where prevalence of ear rot was already observed, a decrease of 78% in disease incidence was noticed when PFJM1 bioformulation was applied to field. The field treated with PFJM1 showed increment in all growth parameters (**Table 14** and **Figure 50**). In comparison to untreated seed, seed coated with PFJM1 bioformulation showed enhancement in germination by 28%. The shoot and root length and fresh and dry weight of plant increased by 24%, 104%, 53% and 36% respectively. However, increment in fresh and dry weight of ear were found 95% and 156% over control. Further these was increase of yield in terms of seed per plant and 100 grain weight were recorded 128 % and 154% respectively. Results also indicated that in comparison to thiram treated seed, PFJM1 bioformulation treatment increased significantly in all growth parameters tested. Here shoot and root length were increased by 7% and 70%, plants fresh and dry weight by 4% and 24 %. However, increment in ear fresh and dry weight, number of seed and 100 grain weight were recorded to increase by 45 %, 82%, 23% and 65% respectively.

TABLES AND FIGURES



5. TABLES AND FIGURES

Table 1. Details of isolates

S.No.	Name of Place	Name of Isolates	Name of plants
1.	Sitapur	PFS1, PFS2, PFS3, PFS4	Maize (<i>Z. mays</i> L.)
		PFS5, PFS6, PFS7, PFS8	Mint (<i>Mentha spicata</i> L.)
		PFS9, PFS10	Rape seed (<i>Brassica napus</i> L.)
2.	Hardoi	PFH1, PFH2, PFH3, PFJM1, PFH5, PFH6, PFH7, PFH8, PFH9, PFH10	Maize (<i>Z. mays</i> L.)
3.	Lucknow	PFL1, PFL2, PFL3, PFL4	Maize (<i>Z. mays</i> L.)
		PFL5, PFL6, PFL7	Spinach (<i>Spinacia oleracea</i> L.)
		PFL8, PFL9, PFL10	Tomato (<i>Solanum lycopersicum</i> L.)
4.	Ballia	PFB1, FB2, FB3, PFB4, PFB5, PFB6, PFB7, PFB8, PFB9, PFB10	Maize (<i>Z. mays</i> L.)
5.	Safedabad	PFS1, PFS2	Wheat (<i>Triticum aestivum</i> L.)

Table 2. Phenotypic characteristics of isolates

Name of Isolates	Gram reaction	Colony morphology	Shape	Motility	Pigment production
PFS1	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS2	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS3	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS4	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS5	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS6	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS7	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS8	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS9	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS10	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFH1	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFH2	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSH3	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSJM1	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSH5	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSH6	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSH7	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSH8	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFSH9	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFH10	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL1	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL2	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL3	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL4	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL5	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL6	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL7	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL8	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL9	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFL10	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB1	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB2	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB3	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB4	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB5	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB6	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB7	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB8	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB9	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFB10	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS1	-	Slimy mucoid	Curved rod	+	Yellowish-green
PFS2	-	Slimy mucoid	Curved rod	+	Yellowish-green

+ = Positive for trait; - = Negative for trait

Table 3. Biochemical characteristics of isolates

Name of Isolates	Oxidase	Catalase	Citrate	Urease	Indole	MR -VP	OF	Levan	Gelatinase	Arginine dihydrolase	Starch hydrolysis	Casein hydrolysis	Lipase	Growth at	
														4°C	42°C
PFS1	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFS2	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFS3	+	+	+	+	-	-	O	+	+	+	-	-	+	-	-
PFS4	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFS5	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFS6	+	+	+	+	-	-	O	+	+	+	-	-	-	+	-
PFS7	+	+	+	+	-	-	O	+	+	+	-	-	+	-	-
PFS8	+	+	+	+	-	-	O	+	+	+	-	+	-	-	-
PFS9	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFS10	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFH1	+	+	+	+	-	-	O	+	+	+	-	+	-	-	-
PFH2	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFSH3	+	+	+	+	-	-	O	+	+	+	+	-	-	-	-
PFSJM1	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFSH5	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFSH6	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFSH7	+	+	+	+	-	-	O	+	-	+	-	-	-	-	-
PFSH8	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFSH9	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFH10	+	+	+	+	-	-	O	+	+	+	+	-	-	-	-
PFL1	+	+	+	+	-	-	O	-	+	+	-	-	-	-	-
PFL2	+	+	+	+	-	-	O	+	+	+	-	+	-	-	-
PFL3	+	+	+	+	-	-	O	+	-	+	-	-	-	-	-
PFL4	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFL5	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFL6	+	+	+	+	-	-	O	-	-	+	+	+	-	-	-
PFL7	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFL8	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFL9	+	+	+	+	-	-	O	-	+	+	-	-	-	-	-
PFL10	+	+	+	+	-	-	O	+	+	+	-	+	-	-	-
PFB1	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFB2	+	+	+	+	-	-	O	-	+	+	-	-	-	-	-
PFB3	+	+	+	+	-	-	O	+	+	+	-	-	-	+	-
PFB4	+	+	+	+	-	-	O	+	-	+	-	-	-	-	-
PFB5	+	+	+	+	-	-	O	+	+	+	+	-	-	-	-
aPFB6	+	+	+	+	-	-	O	+	+	+	-	+	-	-	-
PFB7	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFB8	+	+	+	+	-	-	O	+	+	+	+	-	+	-	-
PFB9	+	+	+	+	-	-	O	+	+	+	-	+	-	-	-
PFB10	+	+	+	+	-	-	O	-	+	+	-	-	-	+	-
PFS1	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-
PFS2	+	+	+	+	-	-	O	+	+	+	-	-	-	-	-

+ = Positive for trait; - = Negative for trait; O = Oxidative

Table 4. Phosphate solubilization activity of isolates

Name of Isolates	Phosphate solubilization index *(PSI)
PFS1	2.137±0.116
PFS2	2.421±0.211
PFS3	3.068±0.089
PFS4	2.336±0.148
PFS5	2.027±0.055
PFS6	1.634±0.068
PFS7	2.582±0.167
PFS8	2.100±0.122
PFS9	2.400±0.108
PFS10	3.132±0.080
PFH1	3.479±0.182
PFH2	2.300±0.088
PFH3	1.123±0.120
PFJM1	4.372±0.088
PFH5	ND
PFH6	ND
PFH7	2.232±0.182
PFH8	0.000±0.000
PFH9	3.170±0.134
PFH10	1.644±0.254
PFL1	3.178±0.126
PFL2	2.484±0.228
PFL3	ND
PFL4	ND
PFL5	3.060±0.122
PFL6	2.236±0.072
PFL7	3.034±0.132
PFL8	ND
PFL9	0.933±0.050
PFL10	ND
PFB1	2.334±0.084
PFB2	2.274±0.093
PFB3	1.001±0.026
PFB4	2.553±0.125
PFB5	2.755±0.140
PFB6	2.133±0.079
PFB7	2.153±0.116
PFB8	2.426±0.070
PFB9	2.082±0.103
PFB10	3.380±0.164
PFSF1	2.975±0.030
PFSF2	2.136±0.127

*Values are mean of triplicates; ND (Not detectable); ± (SD)

Table 5. Zinc solubilization activity of isolates

Name of Isolates	Zinc solubilization index* (ZSI)
PFS1	1.960±0.055
PFS2	1.129±0.106
PFS3	2.173±0.133
PFS4	1.705±0.187
PFS5	0.955±0.050
PFS6	0.879±0.081
PFS7	0.987±0.183
PFS8	0.946±0.056
PFS9	1.145±0.050
PFS10	1.523±0.132
PFH1	2.020±0.106
PFH2	ND
PFH3	ND
PFJM1	3.213±0.141
PFH5	ND
PFH6	ND
PFH7	1.131±0.106
PFH8	ND
PFH9	2.543±0.249
PFH10	0.449±0.096
PFL1	2.242±0.128
PFL2	1.618±0.090
PFL3	ND
PFL4	ND
PFL5	2.462±0.103
PFL6	1.752±0.153
PFL7	2.108±0.060
PFL8	ND
PFL9	0.327±0.069
PFL10	ND
PFB1	1.174±0.099
PFB2	2.854±0.460
PFB3	2.150±0.126
PFB4	0.651±0.122
PFB5	0.484±0.068
PFB6	0.198±0.100
PFB7	0.666±0.057
PFB8	0.587±0.104
PFB9	1.548±0.187
PFB10	2.585±0.099
PFSF1	2.697±0.126
PFSF2	1.858±0.116

*Values are mean of triplicates; ND (Not Detectable); ± (SD)

Table 6. IAA production by isolates

Name of Isolates	IAA production* (mg/l)
PFS1	8.450±0.480
PFS2	10.473±0.471
PFS3	13.557±0.506
PFS4	11.442±0.605
PFS5	9.812±0.209
PFS6	7.757±0.332
PFS7	6.255±0.413
PFS8	12.315±0.475
PFS9	12.250±0.807
PFS10	13.517±0.544
PFH1	0.706±0.033
PFH2	0.271±0.009
PFH3	0.642±0.082
PFJM1	23.190±0.545
PFH5	0.223±0.015
PFH6	0.290±0.283
PFH7	0.793±0.050
PFH8	0.067±0.046
PFH9	13.932±0.403
PFH10	2.047±0.063
PFL1	18.241±0.706
PFL2	10.430±0.632
PFL3	0.199±0.032
PFL4	0.192±0.135
PFL5	14.293±0.663
PFL6	1.131±0.078
PFL7	5.168±0.248
PFL8	0.116±0.018
PFL9	2.021±0.030
PFL10	0.060±0.012
PFB1	8.313±0.358
PFB2	12.625±0.924
PFB3	15.368±0.785
PFB4	3.282±0.111
PFB5	1.055±0.059
PFB6	4.186±0.140
PFB7	2.643±0.156
PFB8	5.237±0.294
PFB9	13.731±0.917
PFB10	16.324±0.716
PFSF1	21.538±0.990
PFSF2	13.716±0.597

*Values are mean of triplicates; ± (SD)

Table 7. GA production by isolates

Name of Isolates	Gibberellic Acid production (mg/l)
PFS1	1.357±0.200
PFS2	1.786±0.062
PFS3	2.101±0.076
PFS4	2.202±0.035
PFS5	0.891±0.033
PFS6	0.783±0.097
PFS7	1.250±0.054
PFS8	0.773±0.025
PFS9	2.080±0.047
PFS10	1.635±0.057
PFH1	2.409±0.199
PFH2	2.041±0.051
PFH3	0.492±0.033
PFJM1	4.353±0.138
PFH5	1.418±0.010
PFH6	0.328±0.174
PFH7	0.493±0.159
PFH8	0.133±0.068
PFH9	3.129±0.103
PFH10	0.670±0.046
PFL1	3.089±0.040
PFL2	2.629±0.073
PFL3	1.561±0.055
PFL4	0.093±0.046
PFL5	2.266±0.044
PFL6	0.712±0.109
PFL7	0.421±0.044
PFL8	1.333±0.060
PFL9	0.895±0.047
PFL10	0.538±0.074
PFB1	2.064±0.058
PFB2	3.327±0.202
PFB3	2.184±0.164
PFB4	1.660±0.087
PFB5	0.206±0.011
PFB6	0.534±0.080
PFB7	0.164±0.059
PFB8	3.501±1.851
PFB9	1.470±0.047
PFB10	2.124±0.072
PFSF1	2.787±0.087
PFSF2	1.731±0.170

*Values are mean of triplicates; ± (SD)

Table 8. Characterization of siderophores by different methods

Name of isolates	Addition of Fe in medium	CAS universal assay		Hydroxymate Absorbance between 400 to 600 nm and peak between 420-450	Catecholate Absorbance of red color developed at 510 nm	Carboxylate		Salicylate Concentration (µg/ml)
		CAS agar assay	CAS liquid assay (% siderophore unit)			Intensity of pink color	Absorption maximum between 190-280nm	
PFJM1	+	+	4.58	+	0.054	+	190.714	1.751
PFH1	+	+	14.41	+	0.039	+	203.990	4.304
PFH8	+	+	14.35	+	0.034	ND	ND	ND
PFS5	+	+	6.850	+	0.021	ND	ND	1.320
PFS3	+	+	6.440	+	ND	ND	ND	ND
PFS7	+	+	7.410	+	0.019	ND	ND	ND
PFL1	+	+	18.290	+	0.042	+	203.990	3.501
PFL3	+	+	15.291	+	ND	ND	ND	ND
PFL5	+	+	14.123	+	ND	ND	ND	ND
PFL6	+	+	14.41	+	ND	ND	ND	ND
PFL7	+	+	ND	ND	ND	ND	ND	ND
PFB4	+	+	ND	ND	ND	ND	ND	ND
PFB7	+	+	ND	ND	ND	ND	ND	ND
PFL9	+	+	11.471	+	0.024	+	226.610	2.501
PFB1	+	+	ND	ND	ND	ND	ND	ND
PFB2	+	+	ND	ND	ND	ND	ND	ND
PFB6	+	+	ND	ND	ND	ND	ND	ND
PFB8	+	+	ND	ND	ND	ND	ND	ND
PFB10	+	+	ND	ND	ND	ND	ND	ND
PFSF1	+	+	36.767	+	0.681	++	223.180	26.512

+ = Positive for trait; ND= Not determine

Table 9. Percent radial growth inhibition of fungi by isolates

Name of Isolates	Percent radial growth inhibition*	
	<i>F. moniliforme</i>	<i>M. phaseolina</i>
PFS1	37.5±0.42	35.30±0.12
PFS2	33.5±0.44	40.30±0.59
PFS3	ND	ND
PFS4	54.74±0.14	60.45±0.48
PFS5	32.78±0.35	65.30±0.40
PFS6	42.50±0.43	32.63±0.01
PFS7	37.50±0.19	37.23±0.03
PFS8	61.11±0.47	47.11±0.24
PFS9	ND	ND
PFS10	33.33±0.23	13.70±0.74
PFH1	27.77±0.04	32.42±0.25
PFH2	3.56±0.86	2.45±0.75
PFH3	31.11±0.75	23.56±0.55
PFJM1	77.33±0.04	69.21±0.23
PFH5	25.23±0.63	27.34±0.48
PFH6	45.72±0.57	36.78±0.96
PFH7	ND	ND
PFH8	33.28±0.34	25.36±0.27
PFH9	42.58±0.55	37.25±0.49
PFH10	7.23±0.46	8.25±0.73
PFL1	12.56±0.95	7.86±0.28
PFL2	42.36±0.91	36.23±0.21
PFL3	56.23±0.73	42.36±0.42
PFL4	18.43±0.47	13.25±0.32
PFL5	ND	ND
PFL6	28.36±0.98	24.86±0.42
PFL7	20.12±0.99	17.23±0.86
PFL8	ND	ND
PFL9	12.30±0.01	23.80±0.84
PFL10	Not shown	Not shown
PFB1	26.20±0.24	21.20±0.23
PFB2	34.30±0.34	33.36±0.10
PFB3	33.00±0.47	22.33±0.32
PFB4	ND	ND
PFB5	ND	ND
PFB6	32.23±0.65	23.14±0.33
PFB7	22.66±0.74	42.3±0.41
PFB8	ND	ND
PFB9	32.25±0.14	39.75±0.25
PFB10	ND	ND
PFSF1	65.27±0.20	59.45±0.24
PFSF2	52.24±0.32	47.43±0.12

Values are mean of triplicates; ND (Not detectable); ± (SD)

Table 10. Functional groups of KBr infrared spectrum of metabolite produced by the strain *P. fluorescens* JM-1

Wavenumbers (cm ⁻¹)	Number of bands	Functional groups
3375–3289	Two	OH, group in alcohols and phenol
2928	One	stretching of CH ₃ compound attached to the benzene ring
1654	One	One sharp band stretching carbonyl group
1545-1400	Three	Aromatic ring stretching
1600–1500	Two	Aromatic ring
1200–1061	Two	C–OH in alcohols
754–604	Two	Alcohols, ethers, acid esters

Table 11. Effect of PFJM1 bioformulation on maize 90 DAS

Treatment	% Seed Germination	Shoot length (cm)	Root length (cm)	Fresh weight of plant (g/plant)	Dry weight of plant (g/plant)	Ear fresh weight (g/plant)	Ear dry weight (g/plant)	Number of seed per plant	100 grain weight
T1	73.33±0.09 ^{ab}	37.32±5.14 ^b	18.37±1.74 ^b	260.81±2.29 ^b	32.27±1.99 ^b	173.98±2.86 ^c	39.66±1.74 ^b	278.33±2.08 ^b	24.435±3.08 ^b
T2	93.33±0.09 ^a	72.20±3.22 ^a	24.68±1.42 ^a	387.59±3.05 ^a	44.75±2.88 ^a	241.37±1.80 ^a	58.14±1.74 ^a	356.33±2.51 ^a	42.582±151 ^a
T3	46.66±0.09 ^c	14.20±2.48 ^{de}	10.39±1.12 ^d	185.50±2.75 ^e	12.22±1.78 ^d	149.08±2.61 ^d	30.64±2.98 ^c	141.66±2.51 ^e	15.291±1.16 ^c
T4	66.66±0.09 ^{bc}	21.07±1.36 ^{cd}	17.73±1.51 ^{bc}	235.97±2.28 ^c	20.10±2.65 ^c	170.02±2.46 ^c	40.68±1.92 ^b	220.33±2.51 ^c	23.896±2.84 ^b
T5	60.00±0.16 ^b	17.20±1.64 ^{cde}	15.55±1.14 ^c	227.90±2.10 ^d	17.44±2.25 ^c	139.33±2.08 ^d	26.44±2.83 ^c	171.09±1.65 ^d	16.688±0.60 ^c
T6	73.66±0.09 ^{bc}	25.02±2.57 ^c	18.53±1.87 ^b	257.87±2.43 ^b	30.65±0.73 ^b	183.66±2.15 ^b	38.28±2.62 ^b	218.33±2.51 ^c	22.933±1.75 ^b

Results are Mean ± SD (n=3). Mean in the column followed by same superscript letters indicate no significant difference (P=0.05) by Duncan's Multiple range test. Three samples were analyzed for each replication and each treatment consisted of three replications.

Table 12. Physicochemical parameters of soil

Physicochemical parameters of soil	Itaunja soil	BBAU green house field
pH	7.953±1.398	8.532±1.236
EC ($\mu\text{C}/\text{cm}$)	137.621±0.712	116.00±1.883
Available P (kg ha^{-1})	35.685±0.495	17.148±0.824
Total N (kg ha^{-1})	125.016±1.025	108.730±0.357
Exchangeable K (kg ha^{-1})	93.885±0.322	82.808±0.140
Organic C (kg^{-1})	2.267±0.821	1.722±1.050

Table 13. Effect of PFJMI bioformulation on maize crop grown in BBAU greenhouse 90 DAS

Treatments	% Seed Germination	Shoot length (cm)	Root length (cm)	Fresh weight of plant (g/plant)	Dry weight of plant (g/plant)	Ear fresh weight (g/plant)	Ear dry weight (g/plant)	Number of seed per plant	100 grain weight
T1	81.23±3.39 ^a	133.133±2.03 ^c	14.93±1.34 ^b	142.50±2.81 ^c	28.26±1.62 ^b	144.69±2.58 ^c	36.98±1.07 ^c	247.33±6.32 ^c	15.95±1.66 ^c
T2	93.17±1.27 ^a	146.333±3.01 ^b	19.03±0.67 ^b	206.65±3.12 ^b	33.77±1.12 ^b	194.35±3.66 ^b	56.01±1.61 ^b	320.00±5.35 ^b	28.89±1.78 ^b
T3	96.82±1.86 ^a	176.50±1.97 ^a	32.30±2.74 ^a	363.50±3.47 ^a	53.41±2.54 ^a	270.57±3.27 ^a	57.81±4.67 ^a	353.66±2.51 ^a	38.24±4.66 ^a

Results are Mean ± SD (n=3). Mean in the column followed by same superscript letters indicate no significant difference (P=0.05) by Duncan's Multiple range test. Three samples were analyzed for each replication and each treatment consisted of three replications.

Table 14. Effect of PFJMI bioformulation on maize crop grown in Itaunja 90 DAS

Treatments	% Seed Germination	Shoot length (cm)	Root length (cm)	Fresh weight of plant (g/plant)	Dry weight of plant (g/plant)	Ear fresh weight (g/plant)	Ear dry weight (g/plant)	Number of seed per plant	100 Grains weight
T1	76.33±3.39 ^b	138.93±2.03 ^c	14.40±1.34 ^b	261.17±2.81 ^c	36.51±1.62 ^b	123.69±2.58 ^c	27.83±1.07 ^c	163.33±6.32 ^c	20.92±1.66 ^c
T2	96.87±1.27 ^a	152.20±3.01 ^b	17.29±0.67 ^b	302.65±3.12 ^b	39.97±1.12 ^b	167.14±3.66 ^b	39.04±1.61 ^b	303.00±5.35 ^b	32.33±1.78 ^b
T3	94.85±1.86 ^a	162.53±1.97 ^a	29.41±2.74 ^d	314.50±3.47 ^a	49.62±2.54 ^a	241.56±3.27 ^a	71.14±4.67 ^a	373.66±2.51 ^a	53.20±4.66 ^a

Results are Mean ± SD (n=3). Mean in the column followed by same superscript letters indicate no significant difference (P=0.05) by Duncan's Multiple range test. Three samples were analyzed for each replication and each treatment consisted of three replications.

Figures

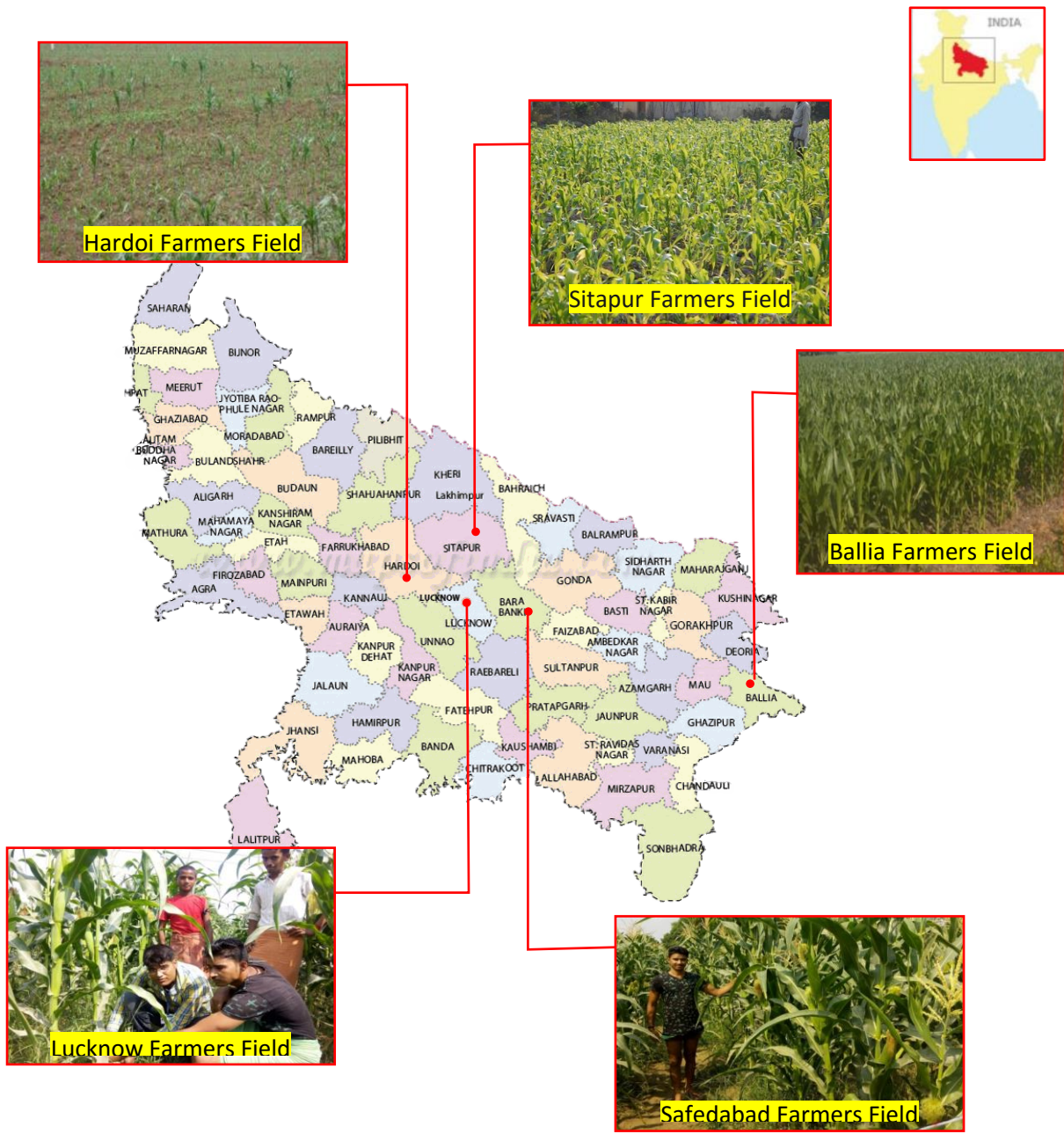


Figure 13. Sampling site locations

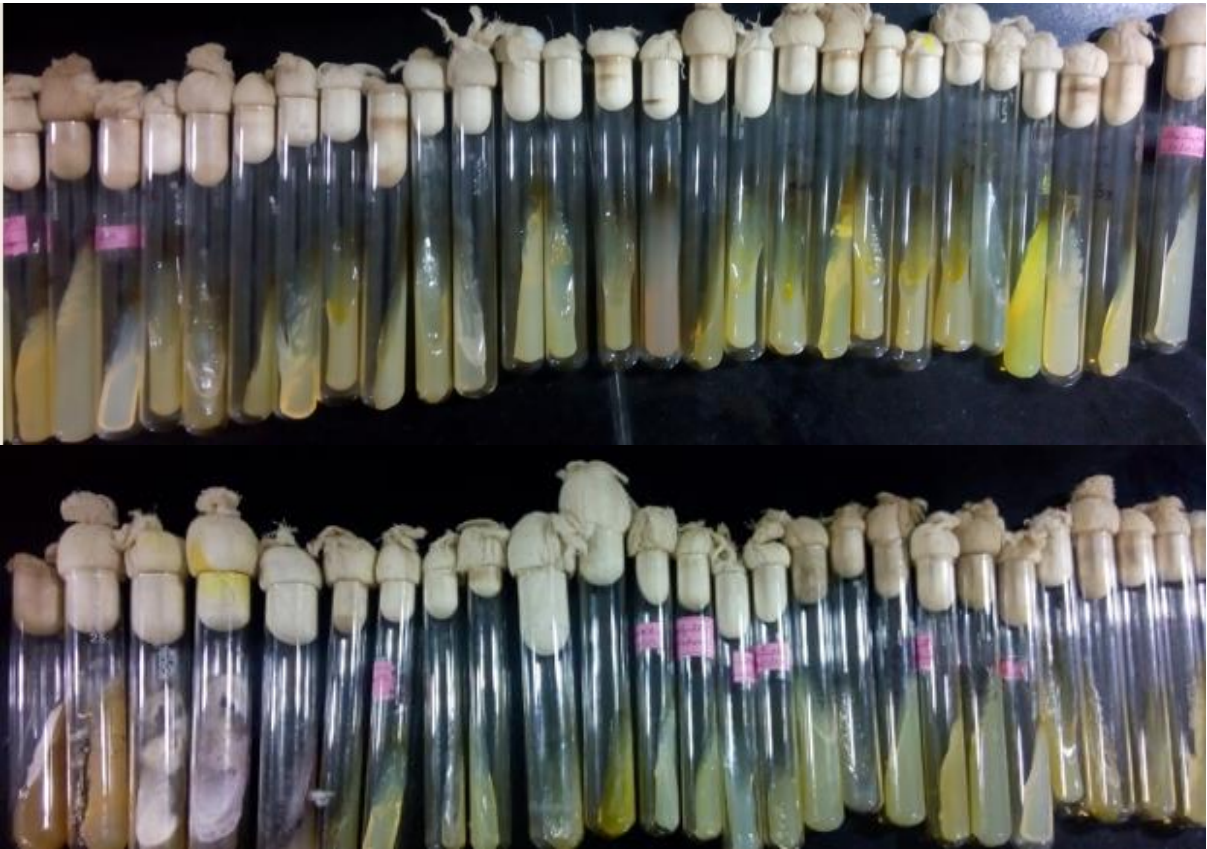


Figure 14. Isolates of fluorescent pseudomonads in KB slants

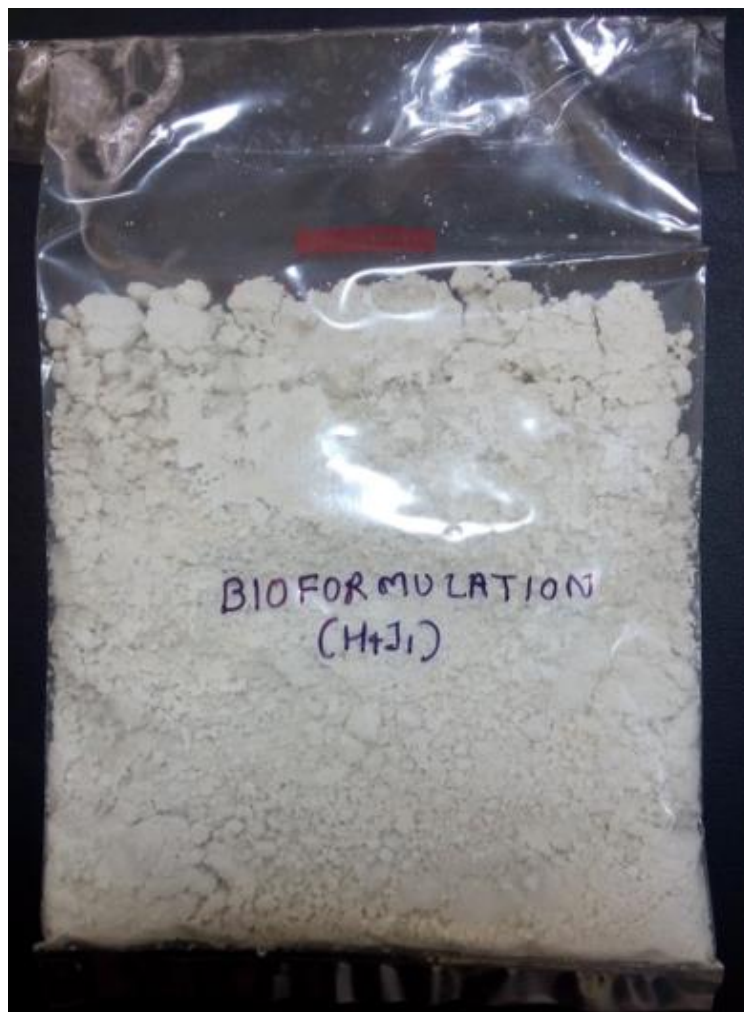


Figure 15. Talc based PFJM1 containing bioformulation



Figure 16. Prevalence of ear rot and fungal diseases in Itaunja field

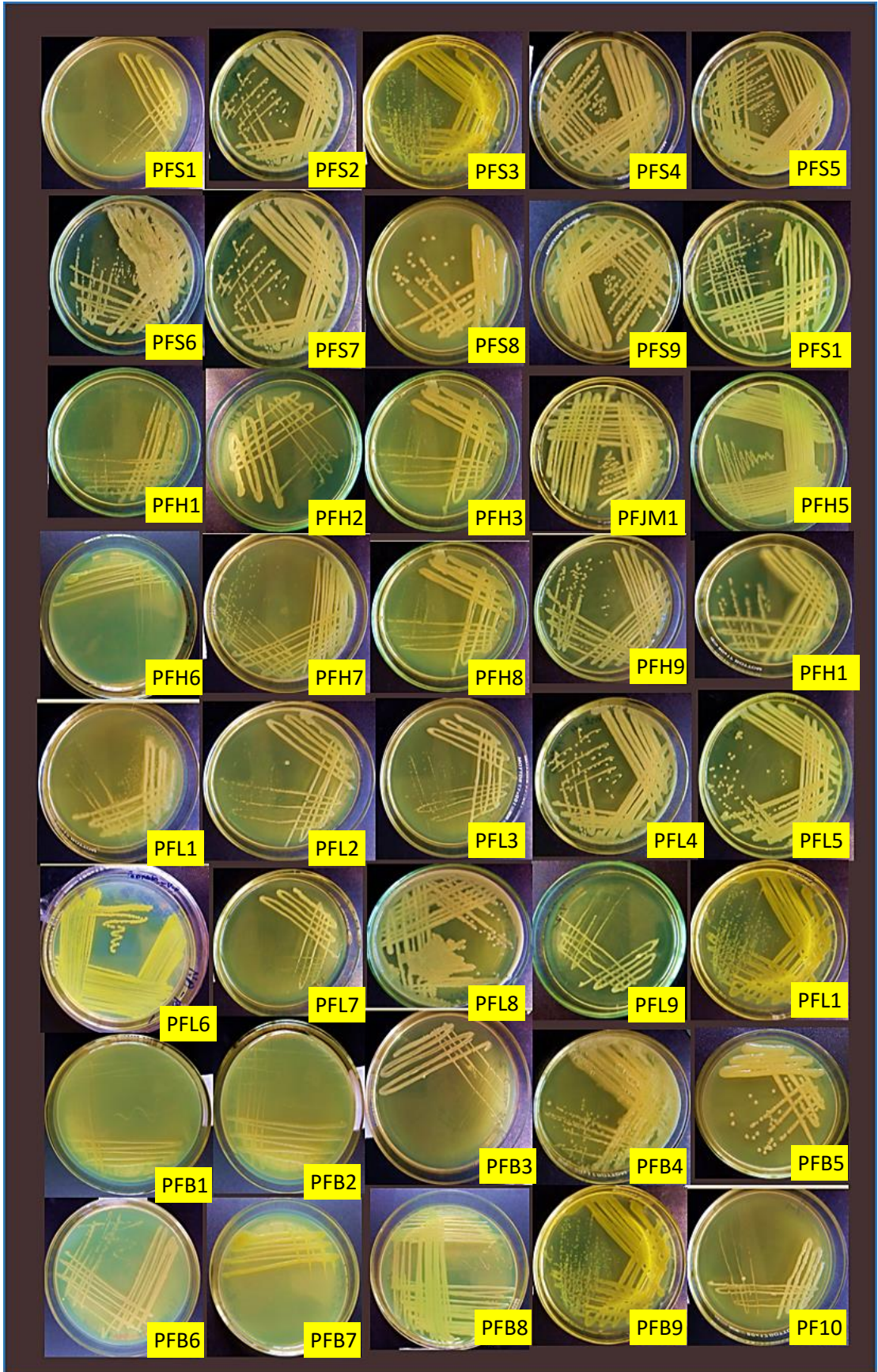
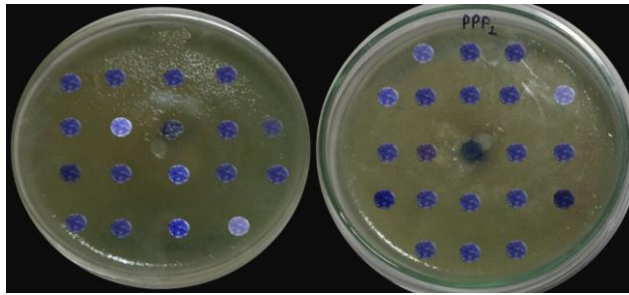
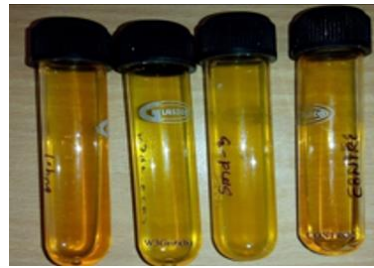


Figure 17. Isolates of fluorescent pseudomonads streaked on KB agar plates



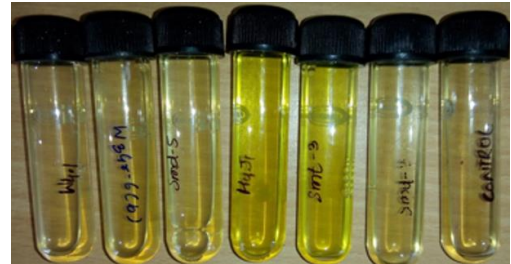
Oxidase



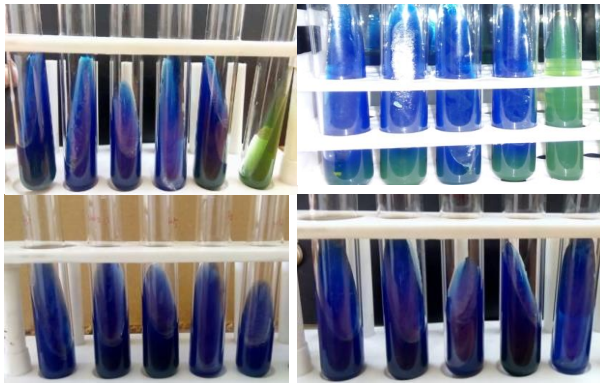
MR test



Catalase test



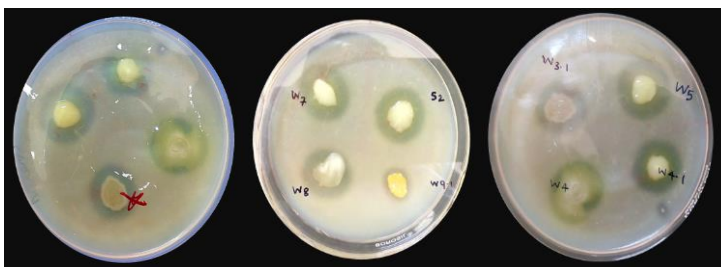
VP test



Citrate utilization test



Motility Indole and lysine test



Casein hydrolysis



Levan

Figure 18. Biochemical tests of isolates

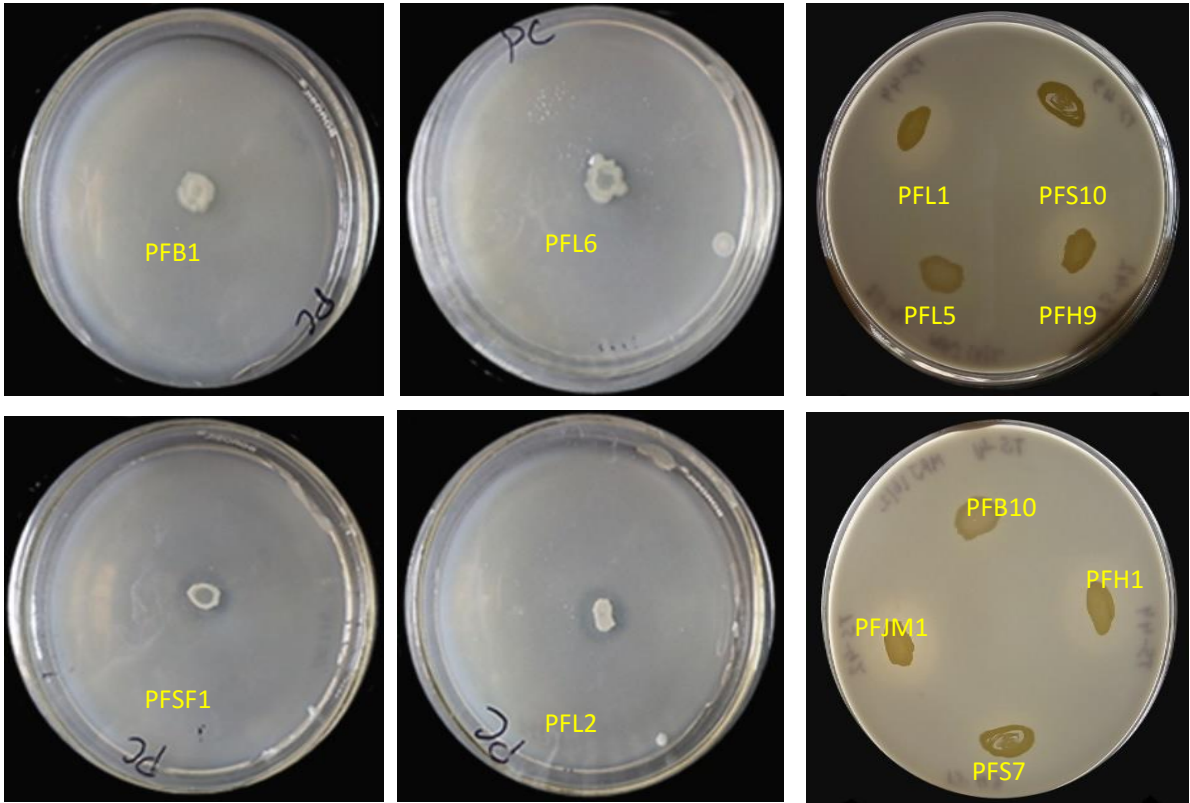


Figure 19. Plates showing phosphate solubilization

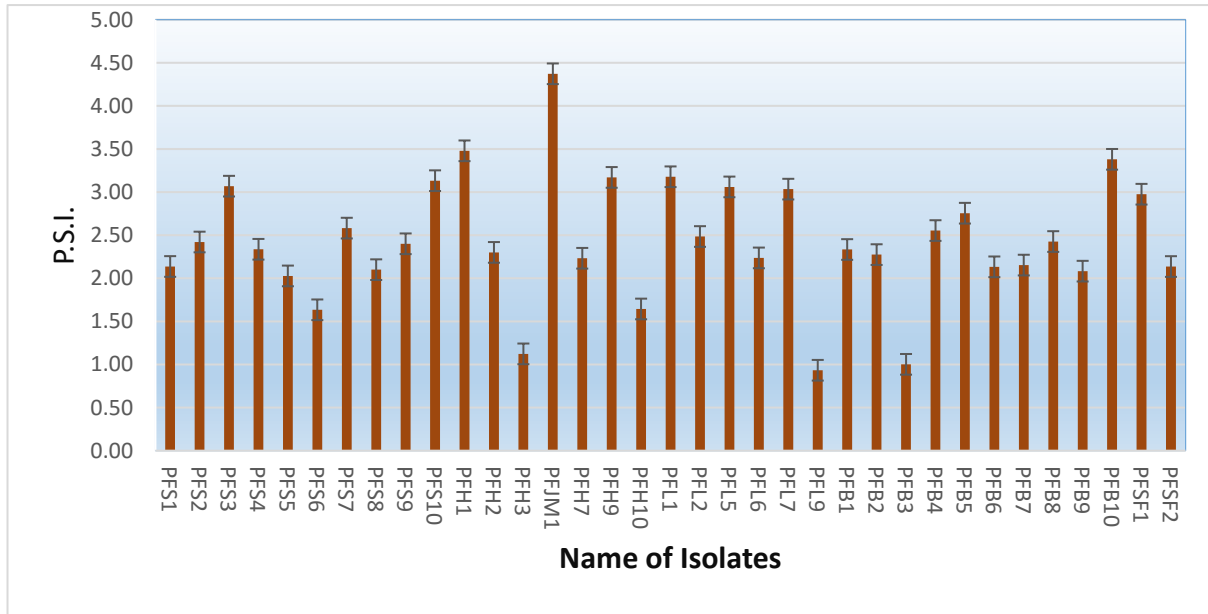


Figure 20. PSI of all isolates. Columns represent means for three replicates (N = 3) with error bars showing standard error

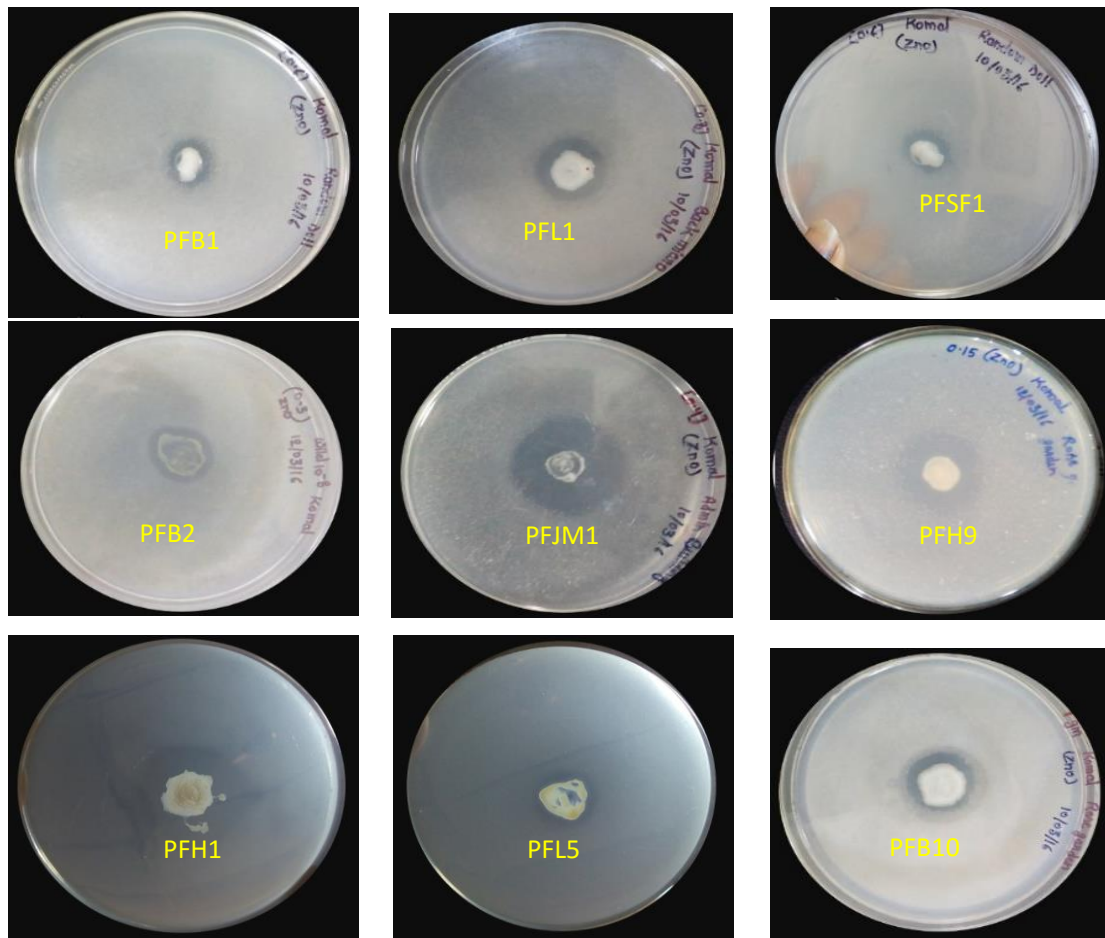


Figure 21. Plates showing Zinc solubilization

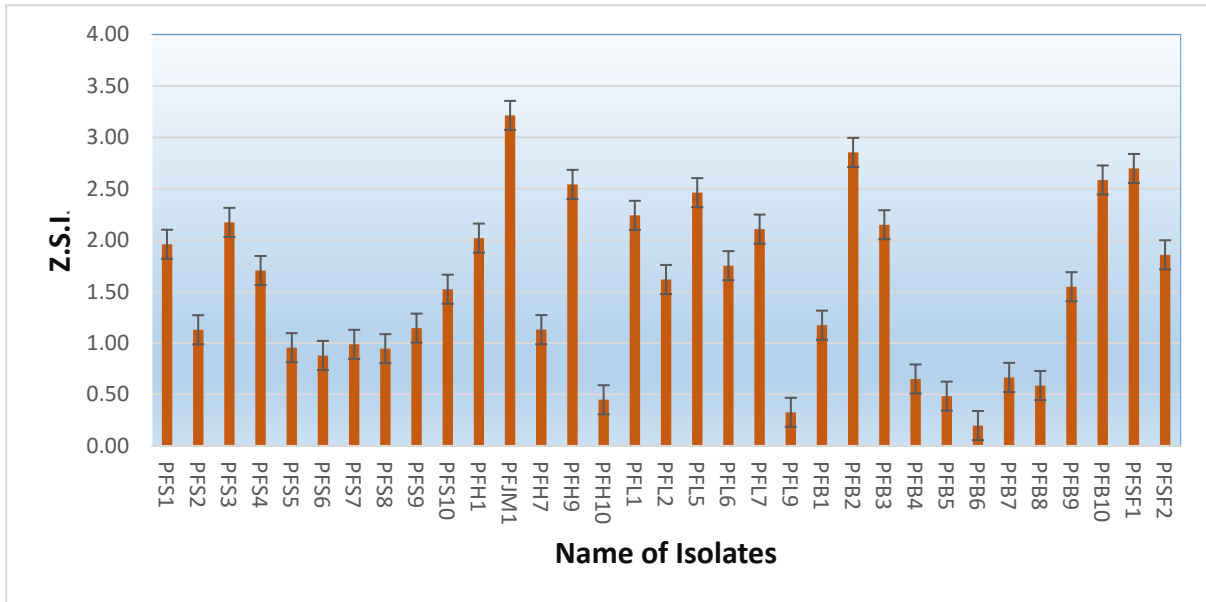


Figure 22. ZSI of all isolates. Columns represent means for three replicates (N = 3) with error bars showing standard error

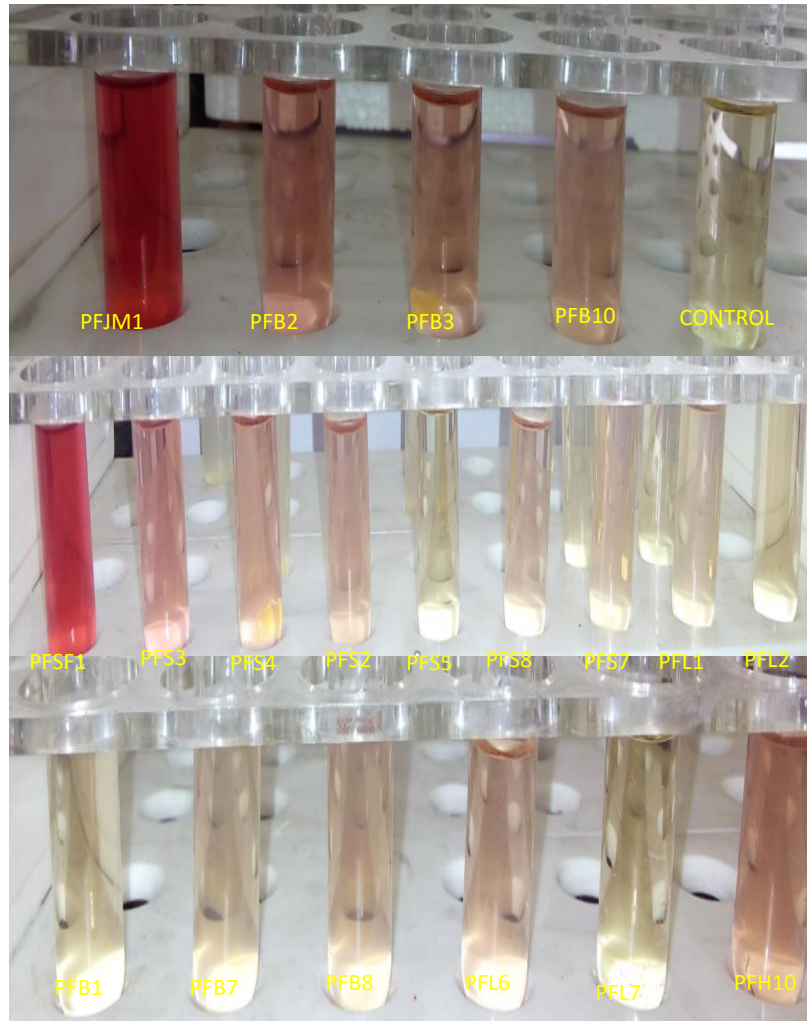


Figure 23. Tubes showing IAA production

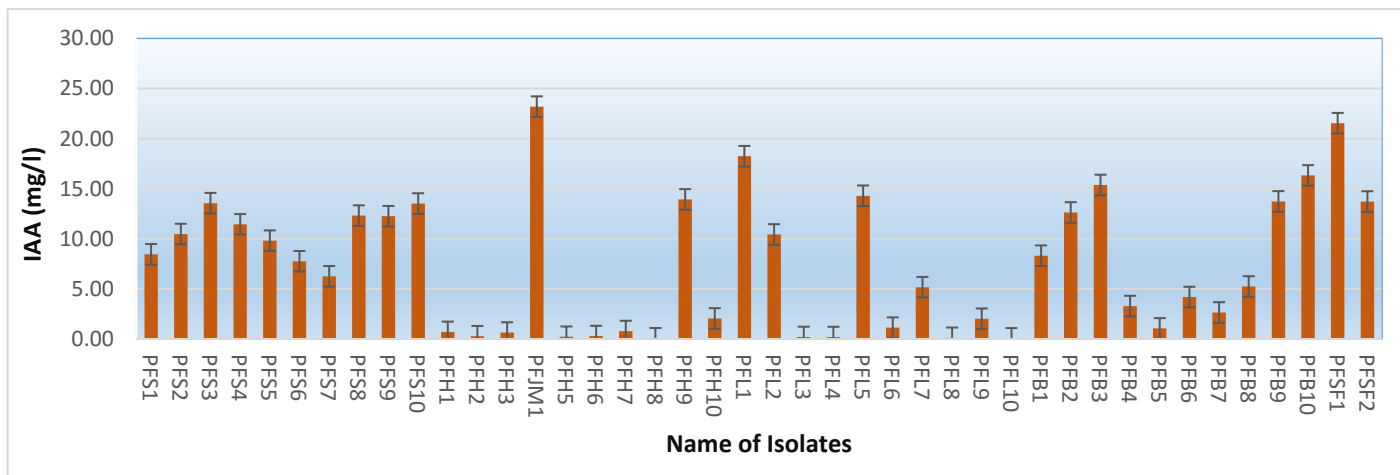


Figure 24. IAA production by all isolates Columns represent means for three replicates (N = 3) with error bars showing standard error

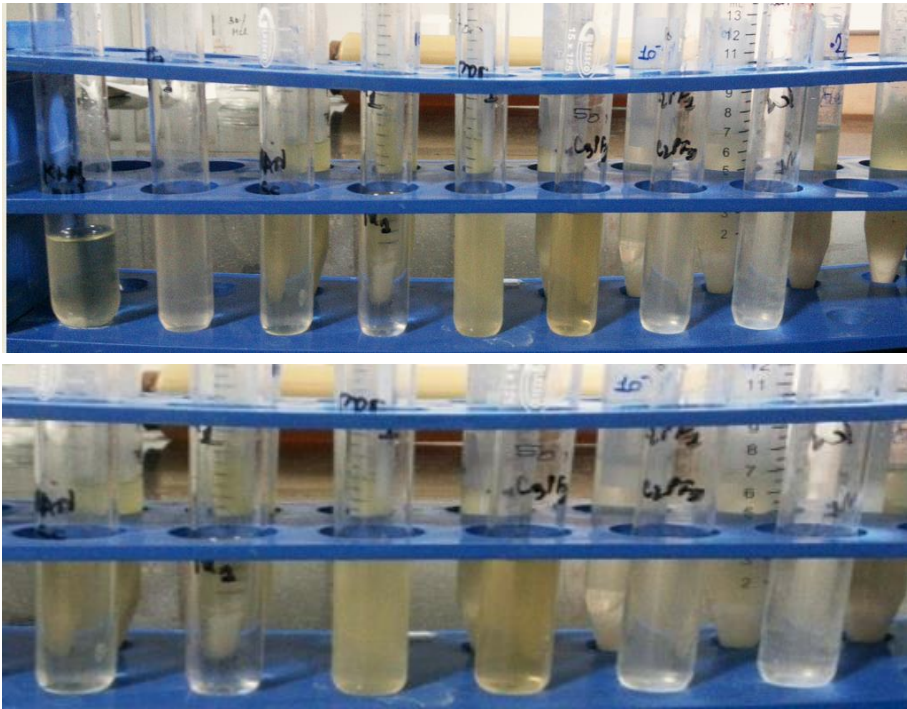


Figure 25. Tubes showing GA production

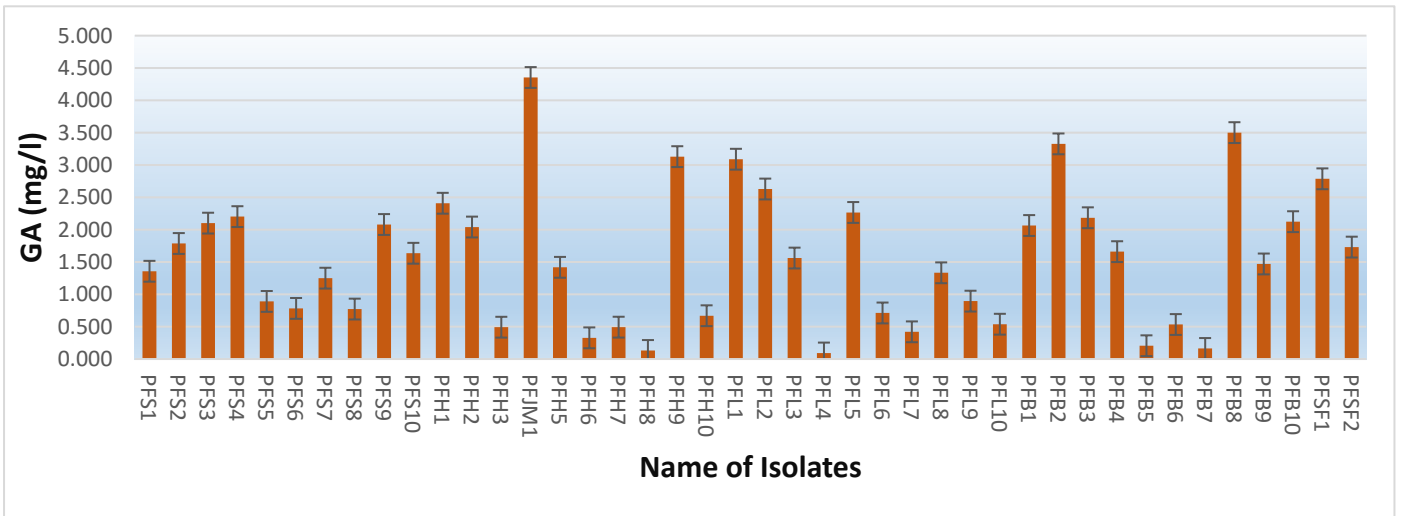


Figure 26. GA production by all isolates Columns represent means for three replicates (N = 3) with error bars showing standard error

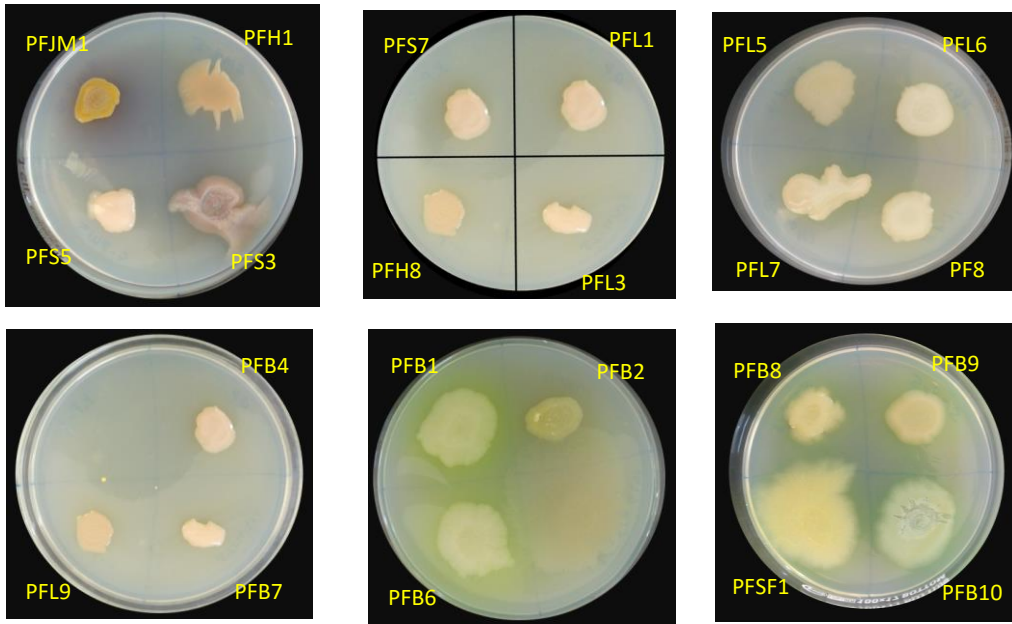


Figure 27. Screening of siderophore producing isolates in Fe containing KB plates

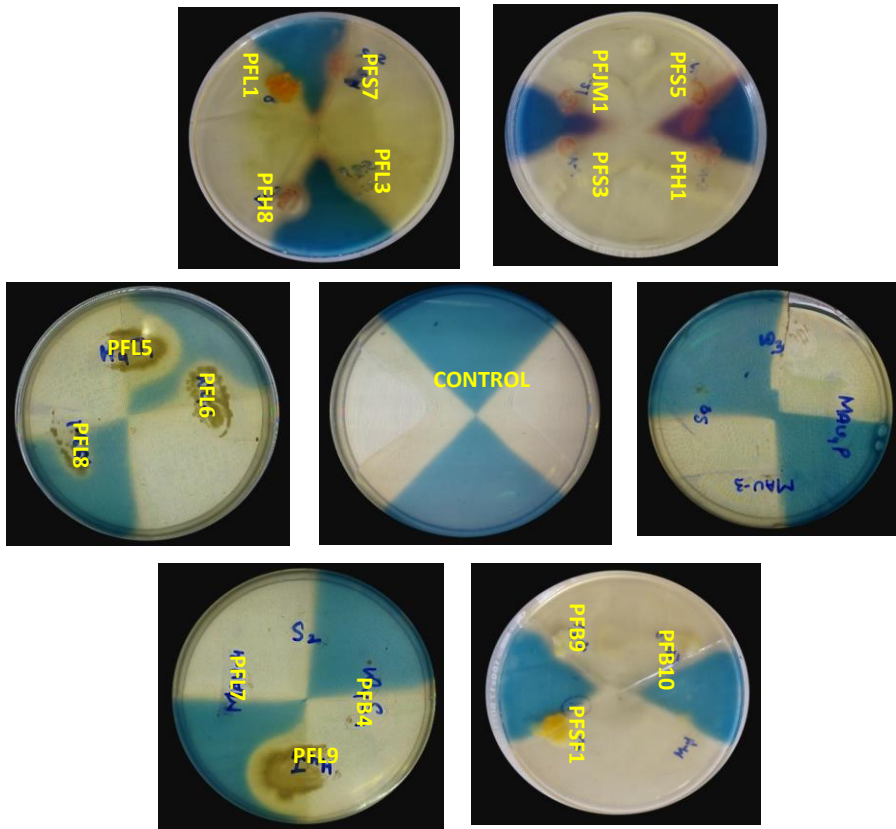


Figure 28. CAS agar plates showing siderophore production

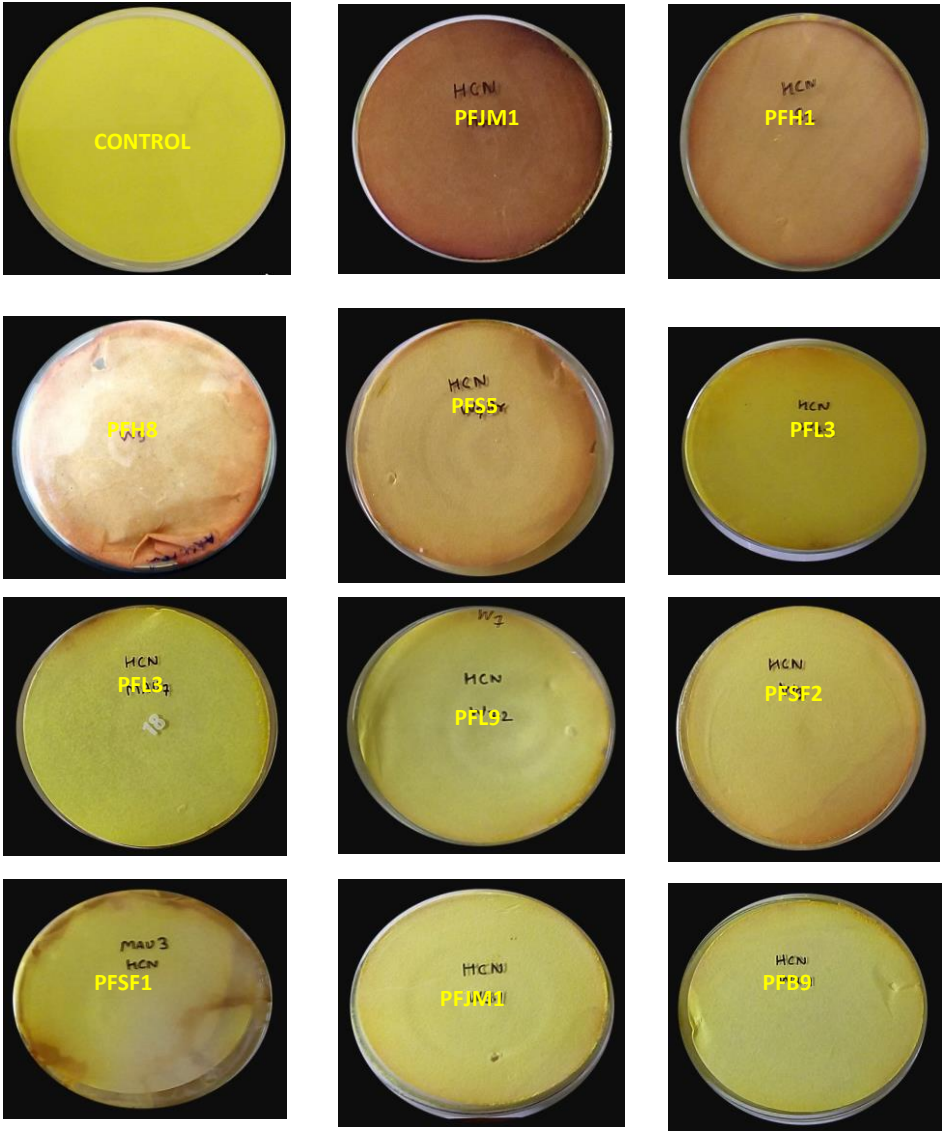


Figure 29. Plates showing HCN production

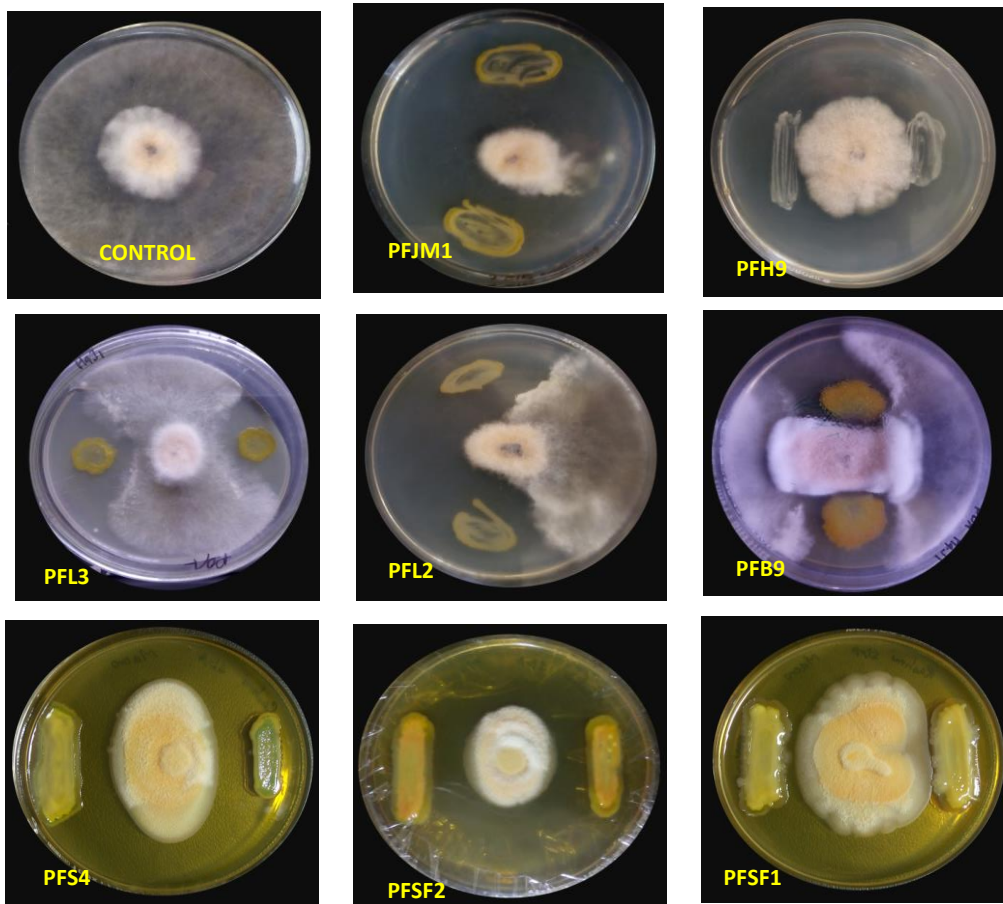


Figure 30. Plates showing in-vitro antifungal activity of some isolates against *M. phaseolina*

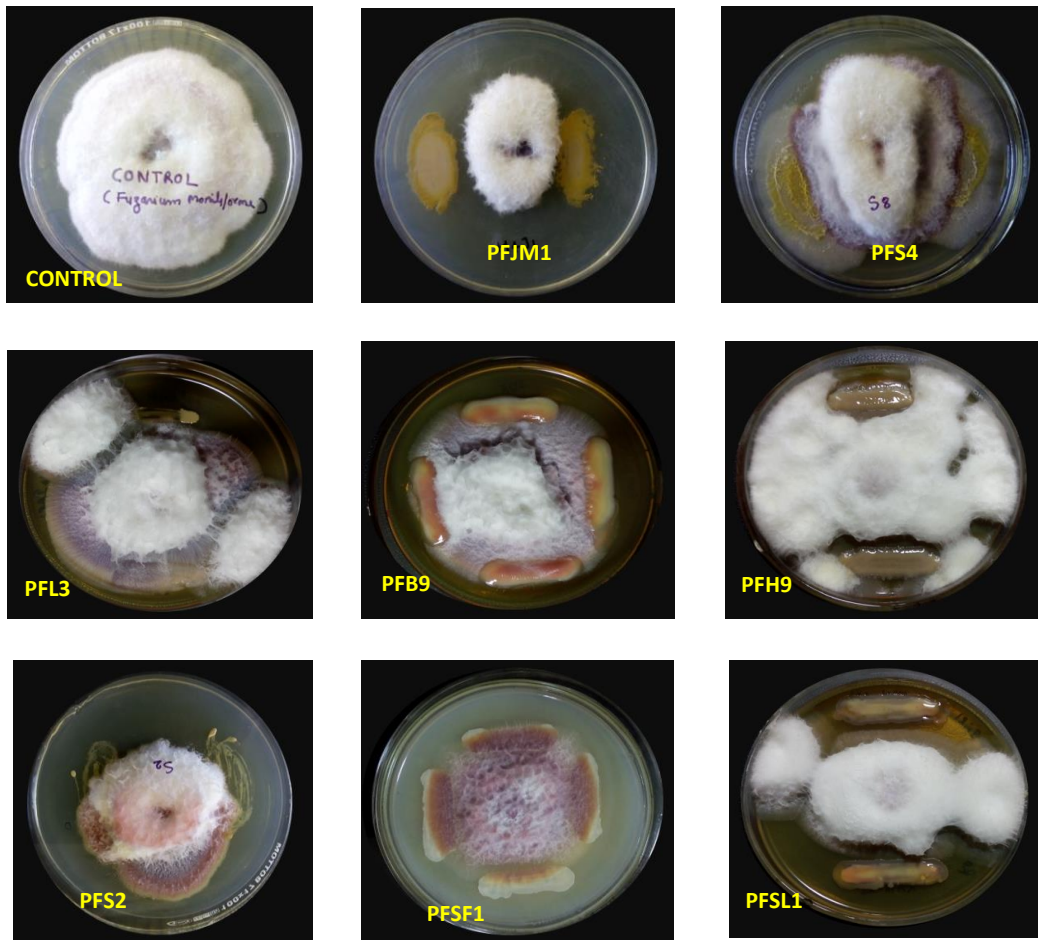


Figure 31. Plates showing in-vitro antifungal activity of some isolates against *F. moniliforme*

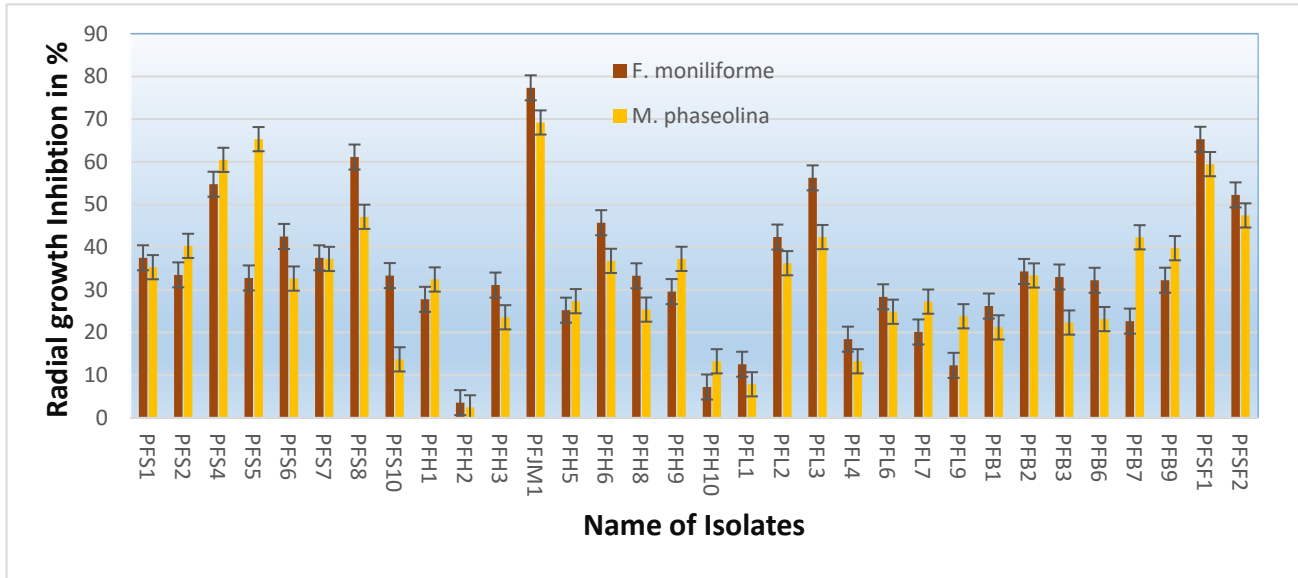


Figure 32. in-vitro antifungal activity of some isolates against *F. moniliforme* and *M. phaseolina*. Columns represent means for three replicates (N = 3) with error bars showing standard error.

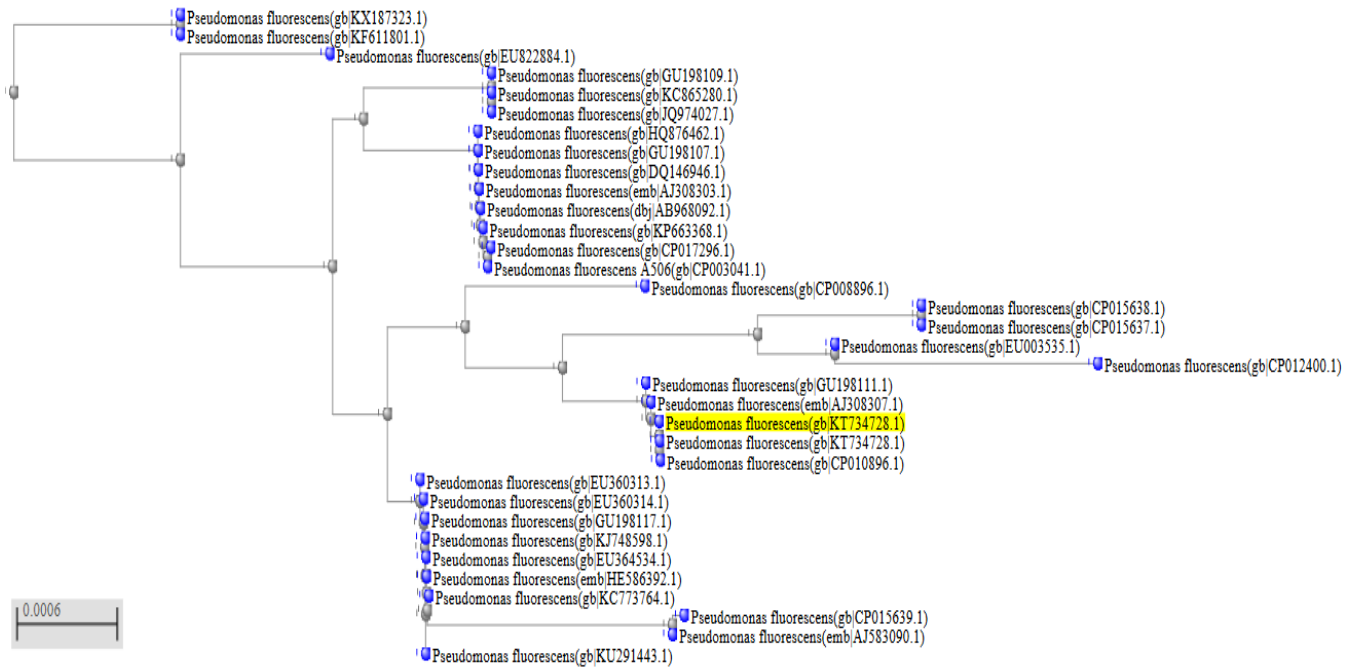


Figure 33. Neighbor joining phylogenetic tree showing relationship of isolate PFJM1 with other fluorescent *Pseudomonas*

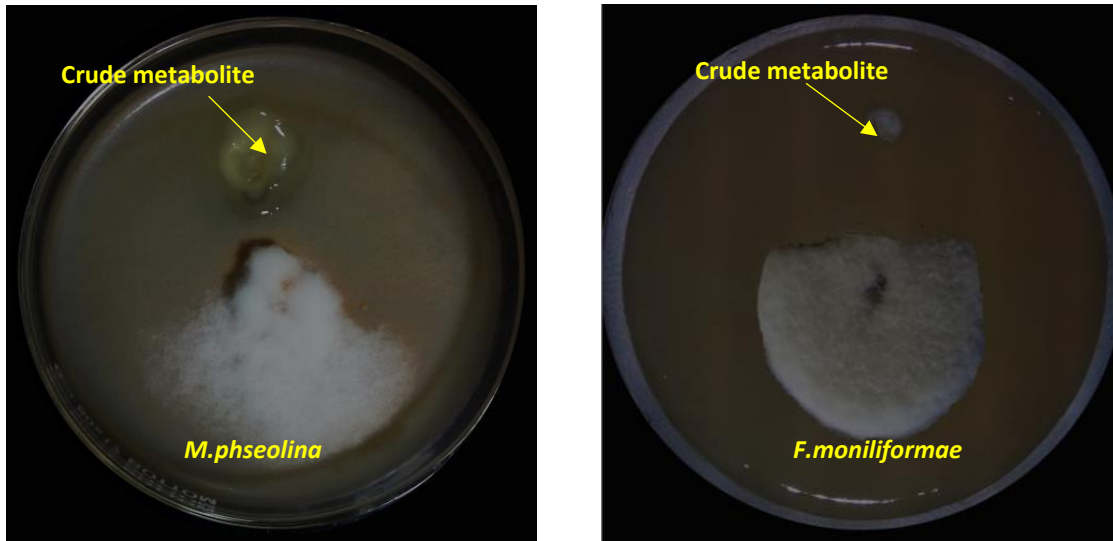


Figure 34. Effect of chloroform extracted crude metabolite from *P. fluorescens* JM-1 on *M. phaseolina* and *F. moniliformae*

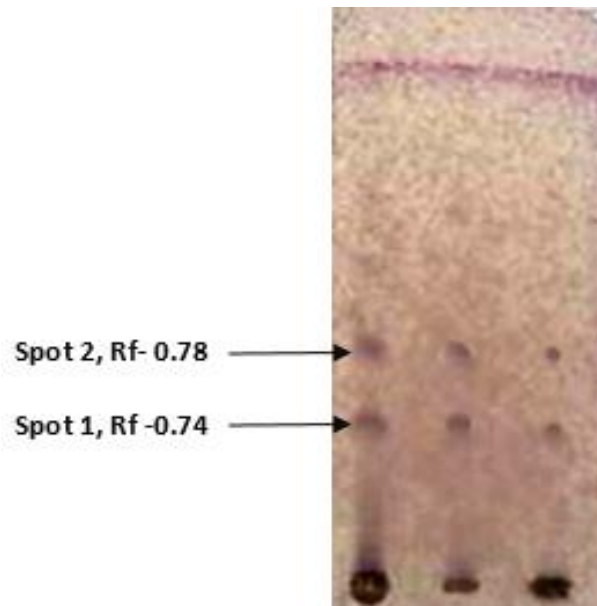


Figure 35. TLC plate showing chloroform extract of crude metabolite from *P. fluorescens* JM-1. Rf value of each compound was marked on plate.

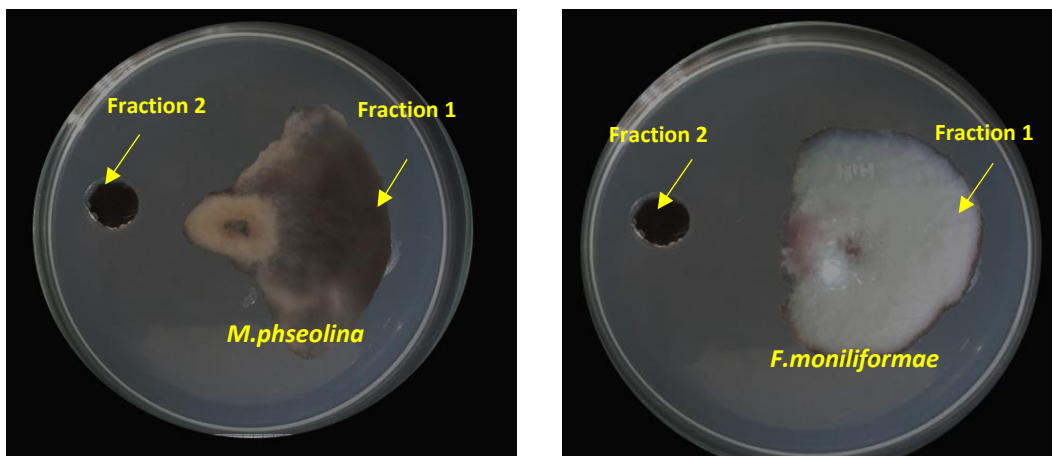


Figure 36. Antifungal activity of TLC fractionated compound on phytopathogen fungi

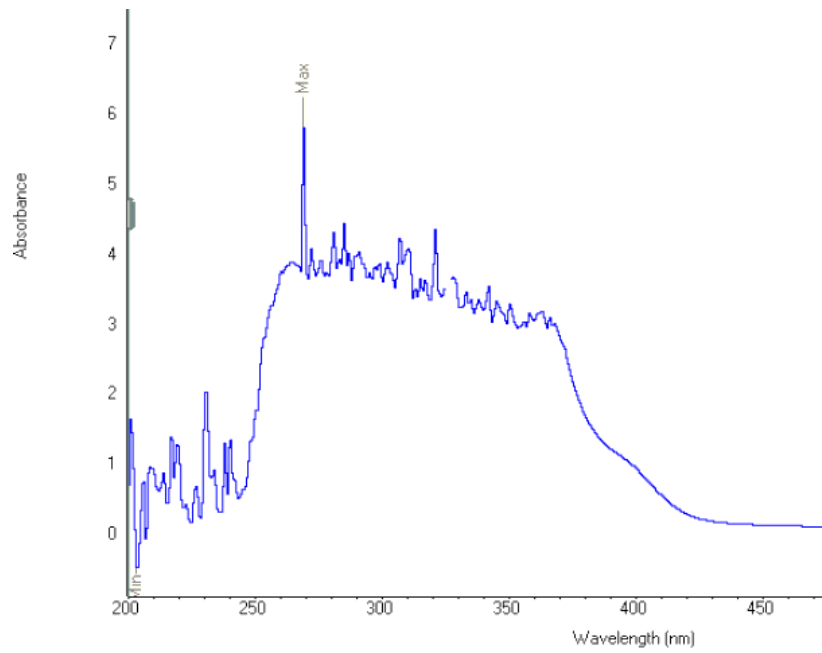


Figure 37. UV-visible spectrum of TLC fractionated compound produced by *P. fluorescens* JM-1

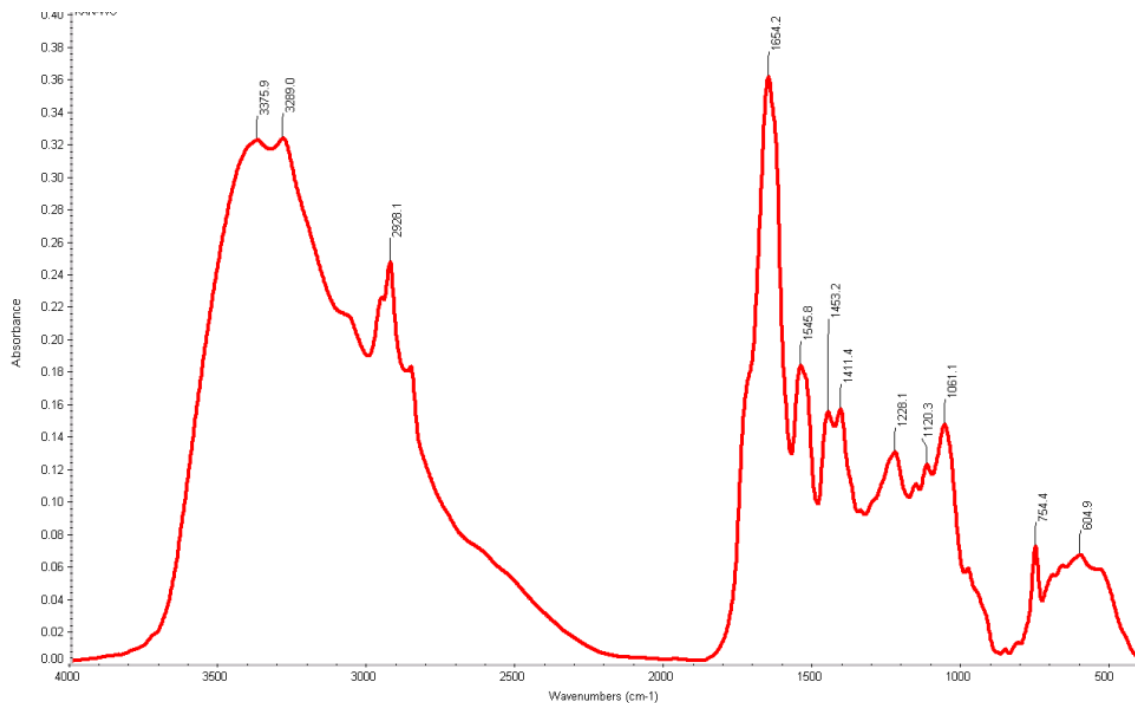
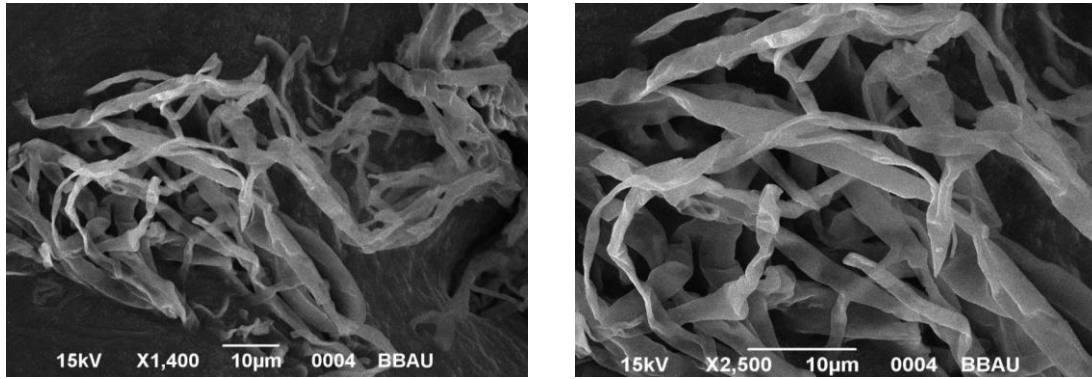
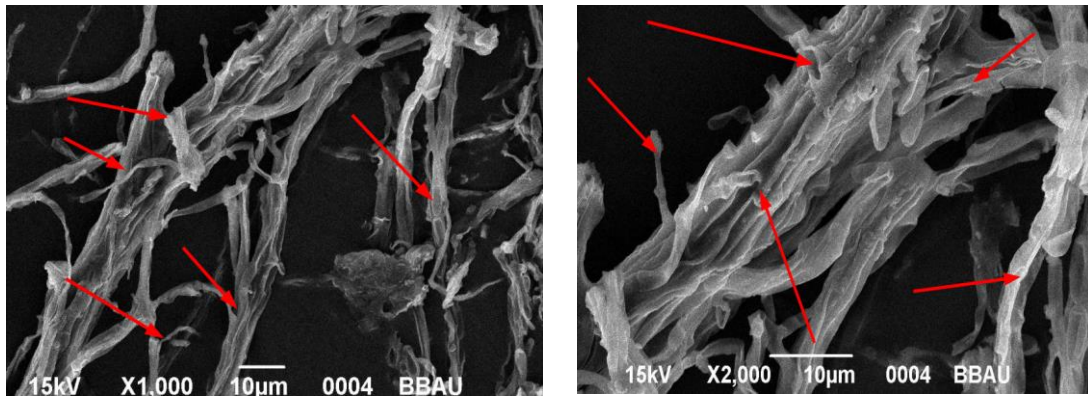


Figure 38. FT-IR spectrum of TLC fractionated compound produced by *P. fluorescens* JM-1

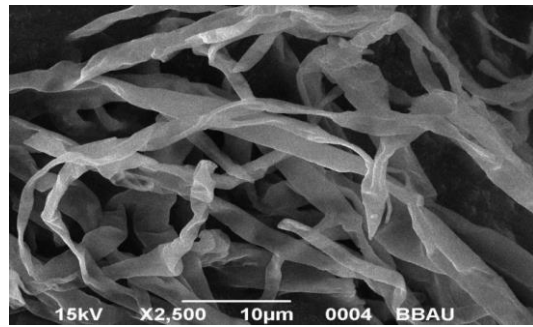
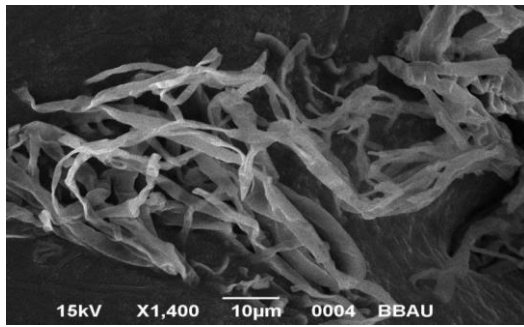


F. moniliforme control

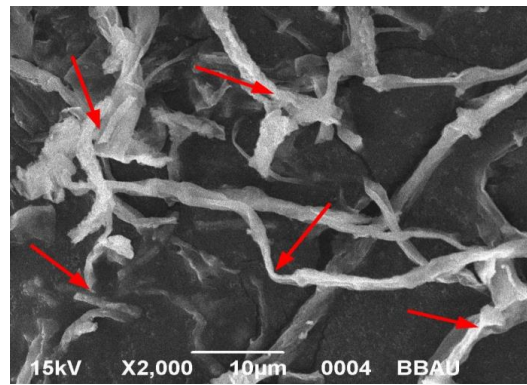
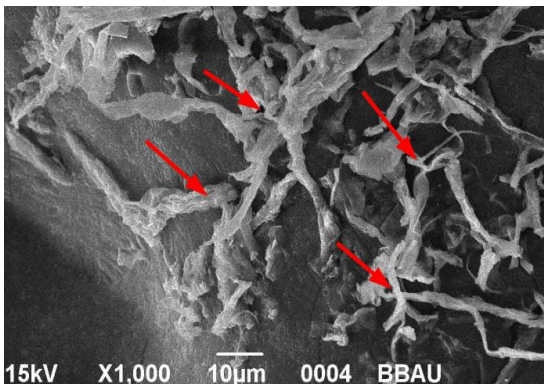


P. fluorescens JM1+ *F. moniliforme*

Figure 39. SEM study showing antagonistic behavior of *P. fluorescens* JM-1 on *M. phaseolina* (arrow indicates site of deformity)

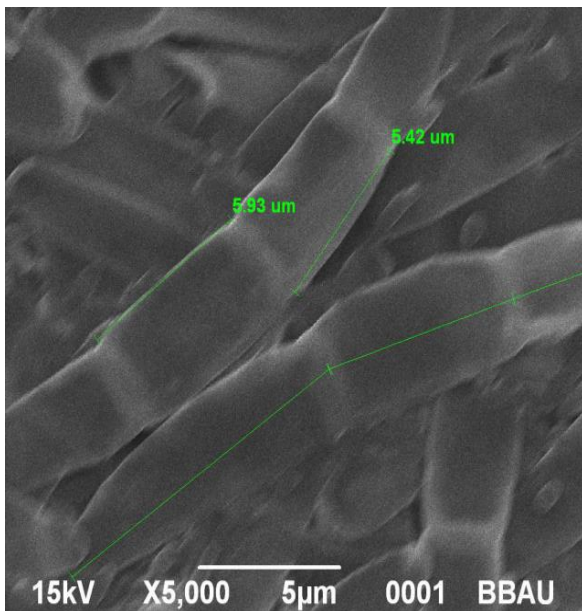


F. moniliforme control

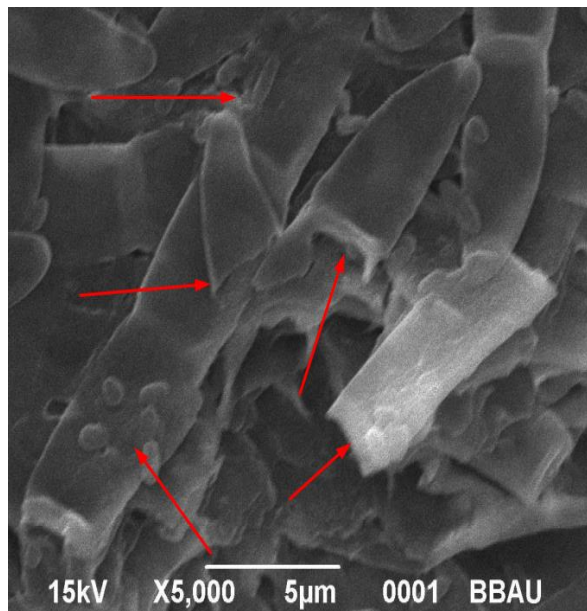


P. fluorescens JM1+*F. moniliforme*

Figure 40. SEM study showing antagonistic behavior of *P. fluorescens* JM-1 on *F. moniliforme* (arrow indicates site of deformity)



Macroconidia (Control)



Macroconidial destruction of *F. moniliforme* by *P.*

Figure 41. SEM study showing *F. moniliforme* macroconidia destruction by *P. fluorescens* JM-1 (arrow indicates site of deformity)

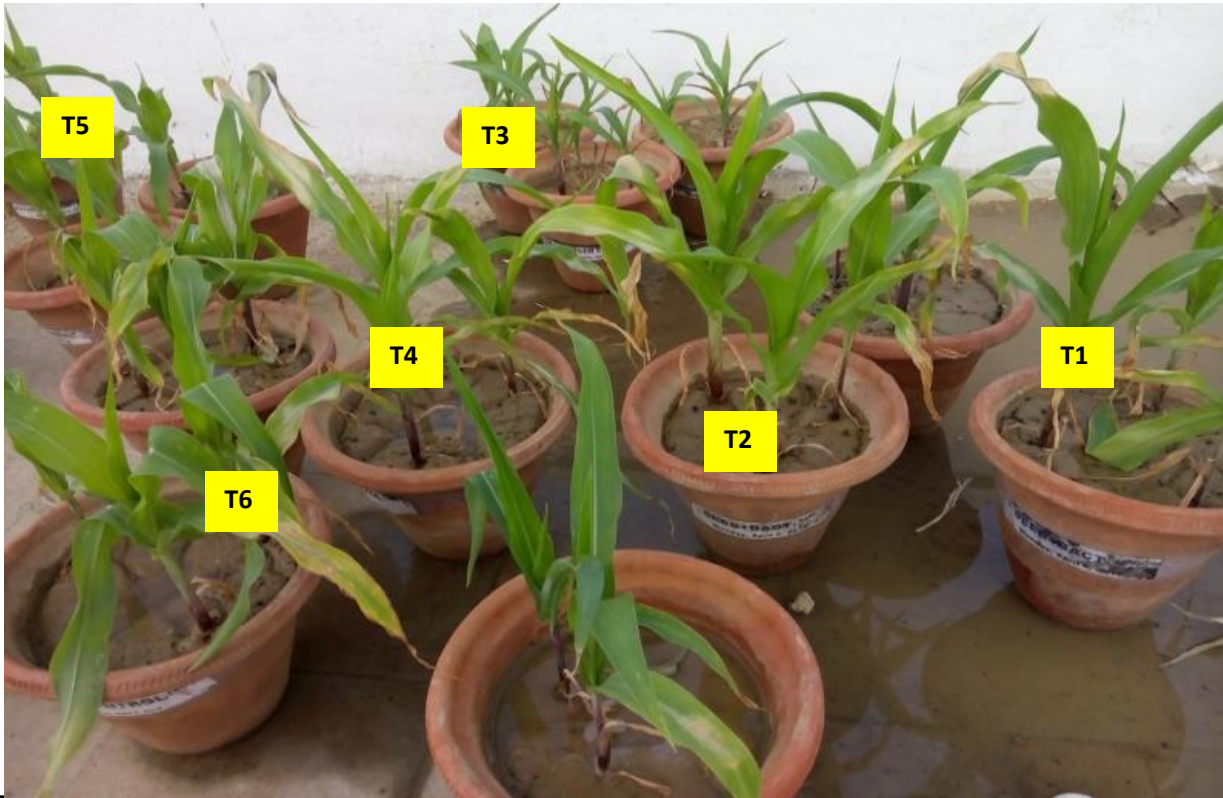


Figure 42. Effect of treatment on plat after 30 DAS



Figure 43. Effect of treatment on plat after 45 DAS



Figure 44. Effect of treatment on plant shoot length

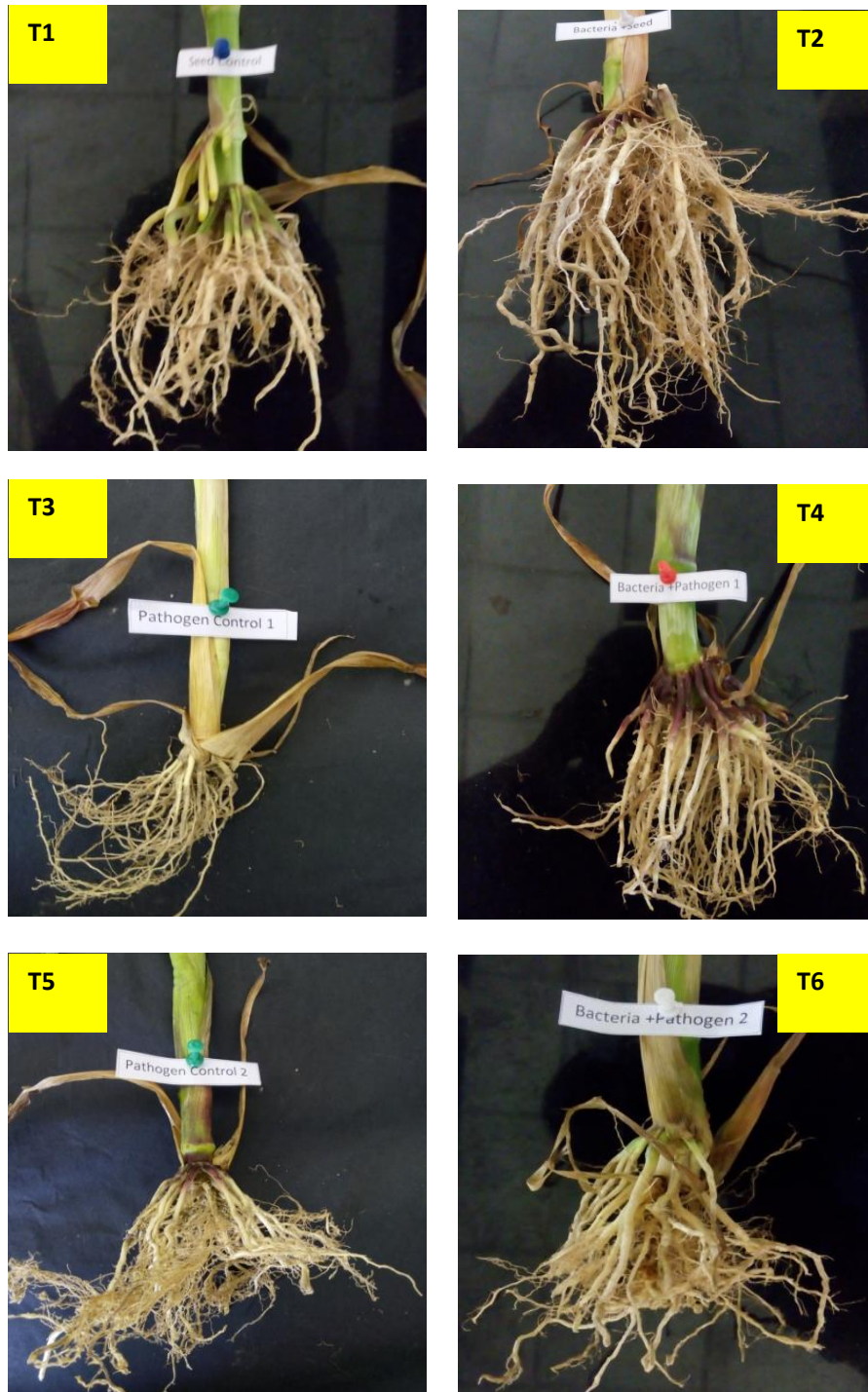


Figure 45. Effect of treatment on root length



Figure 46. Starburst symptoms on maize kernel infected with *F. moniliforme*

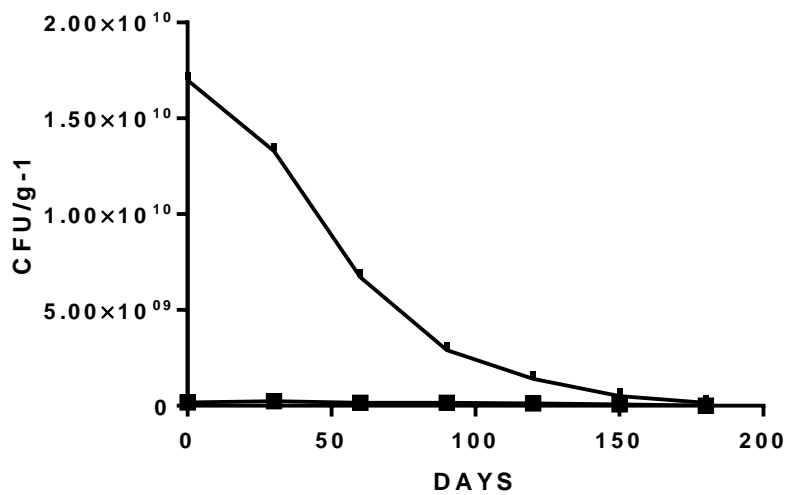


Figure 47. Population density of PFJM1 in talc based bioformulation

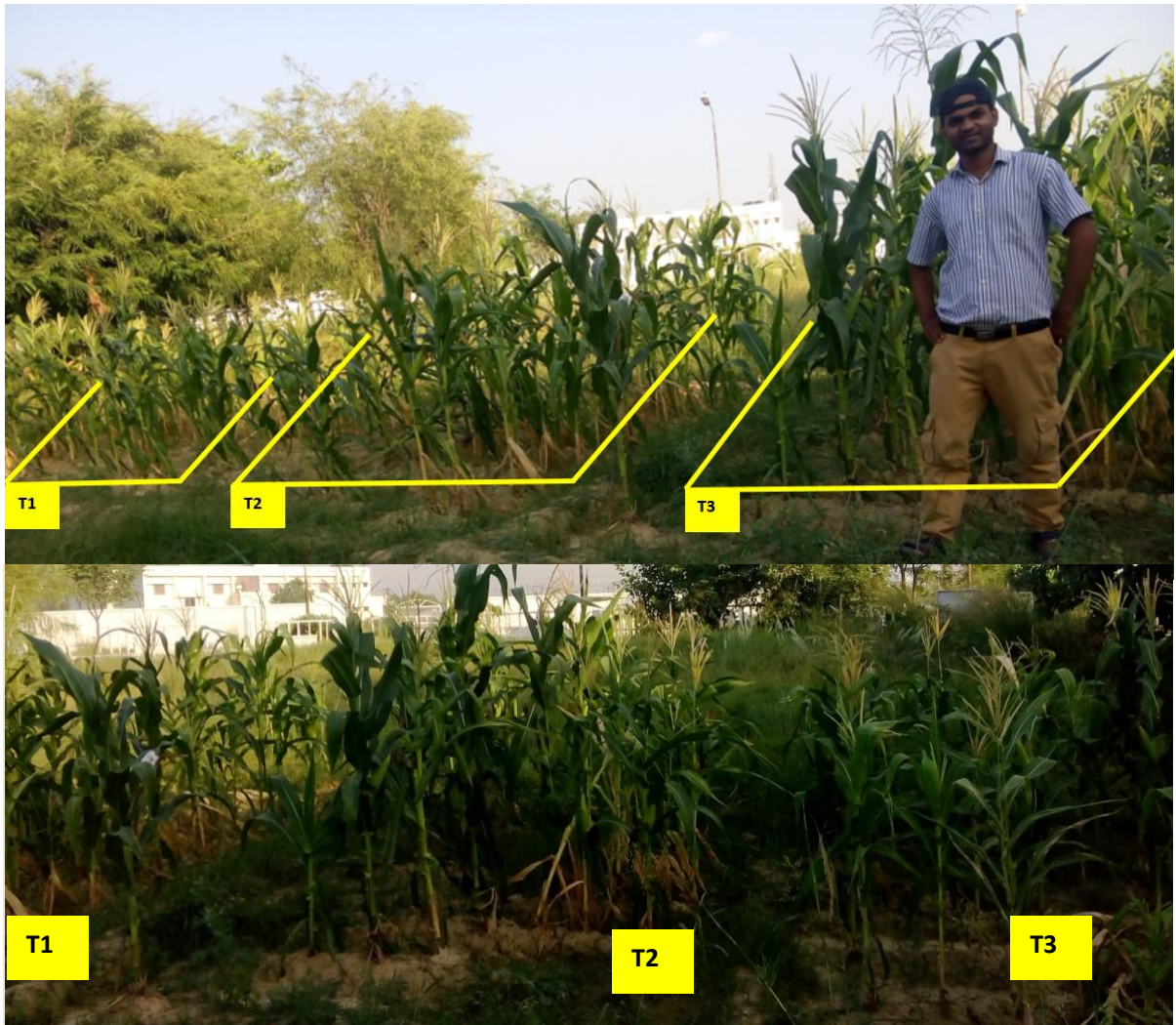


Figure 48. Effect of PFJM1 bioformulation on growth of maize (BBAU field)

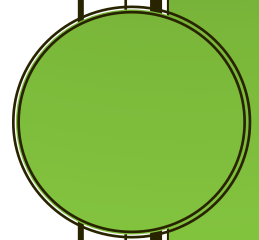


Figure 49. Lodging of stalk due to *M. phaseolina* infection in field



Figure 50. Effect of PFJM1 bioformulation on growth of maize (Itaunja field)

DISCUSSION



6. DISCUSSION

Cereal grains are mainstays of the diets of most of the world's population. Most of the agricultural research being conducted is focused on increasing cereal production to accomplish increasing food needs of the growing world population (Nellemann et al. 2009). Although maize, wheat, and rice, account for 87 % of all grain production, the importance of maize is very high. Area under maize cultivation is also higher than other cereals. For high yield cultivation, maize needs an adequate nutrient supply and protection against diseases. Diseases caused by insects and phytopathogens are serious constraints responsible for loss of half of the maize production in Asia. Crop protection from plant diseases is also very essential in meeting the growing demand for food (Strange and Scott 2005). Much of the success of increasing maize productivity at the global level is the result of the steady use of chemical fertilizers and pesticides. In a study, Tijani and Nurudeen (2012) found that majority of (65%) pesticides used in maize cultivation have adverse effects on soil. Despite the fact that excess use of pesticides is hazardous still they are widely applied, especially in developing countries. In absence of proper usage guidelines and awareness, farmers health is also a major issue (Fajewonyomi 1995). In India, maize farmers perception and awareness towards the harmful effects of pesticides is also negligible. Farmers still use fungicides for seed treatments and/or foliar sprays. Application of beneficial rhizospheric microbes in biocontrol of phytopathogens has been very successful in controlling crop loss (Mishra and Arora 2016). Apart from this, these microbes in the form bioinoculants also showed enhanced growth and minimized reliance on chemical fertilizers (Singh et al. 2016).

In the present study, fluorescent pseudomonads were isolated from the rhizosphere of diverse crops grown in Lucknow and nearby regions. Fluorescent pseudomonads may be identified through examination of the fluorescence "profiles" of diffusible pigments released

into the growth medium (Shelly 1980). All the isolates showed yellow-green pigment production. The yellow-green, water-soluble, fluorescent pigment of the fluorescent pseudomonads known as pyoverdine is regarded as potential taxonomic marker of fluorescent *Pseudomonas* species (Meyer 2000). Morphological appearance (diffused margin and slimy milky mucoid) and physiological and biochemical activities of isolates were checked as described in Bergey's Manual of Systematic Bacteriology (Garrity 2005).

Fluorescent pseudomonads are one of the dominant rhizospheric microbes and have also been reported from cereal crops such as rice, wheat and maize (Naureen et al. 2005; Botelho and Hagler 2006; Fischer 2010). These are one of the best-known PGP microbes (Lawongsa et al. 2008; Fouzia et al. 2015). PGP traits of fluorescent pseudomonads are solubilization of inorganic P, Zn and K minerals, sequestration of Fe and synthesis of phytohormones (Tank and Saraf 2010; Ahemad and Khan 2012; Sivasakthi et al. 2014). In present study, maximum isolates were found to solubilize P and Zn. Amongst all isolates PFJM1 showed maximum amount of P solubilization. Several workers have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds including tri-calcium phosphate, di-calcium phosphate, hydroxyapatite and rock phosphate and confirmed their role in increasing P availability and promote plant (Wakelin et al. 2007; Zaidi et al. 2009; Khan et al. 2010; Sharma et al. 2013). Pal (1998) and Zaida et al. (2003) found an increase in P availability to plants through the inoculation of PSB in pot experiments and under field conditions. In a study Hameeda et al. (2006) tested phosphate-solubilizing ability and other plant growth promoting traits of fluorescent pseudomonads in maize crop. They showed that in glasshouse and field experiments isolate *Pseudomonas* sp. CDB 35 increased in plant biomass by 94%. However, seed treatment with this isolate increased the grain yield by 64% compared to the uninoculated control. Park et al. (2009) proved potential utility of fluorescent pseudomonads for soil amendments to increase soil P

availability or as a biofertilizer. Parani and Saha (2012) also prospected use of phosphate solubilizing *Pseudomonas* as biofertilizer.

Zn is an essential micronutrient required for proper growth of maize. Shabaz et al. (2015) showed that Zn has a significant role in growth and development of maize plants. All the isolates were evaluated for their ability to solubilize Zn and it was observed that 78% isolates showed Zn solubilization in solid medium amended with ZnO. Isolate PFJM1 which solubilized maximum amount of P was also found to solubilize maximum amount of Zn in plate assay. Zn solubilizing ability of fluorescent pseudomonads was studied by Bapiri et al. (2012). Recently Sunithakumari et al. (2016) also found fluorescent pseudomonads as best candidates to solubilize insoluble form of Zn. Although Zn has a vital role in maize health, little work has been done for evaluating role of Zn solubilizing fluorescent pseudomonads in Zn deficient soils as facilitator of Zn micronutrient to maize crops. In a study, Goteti et al. (2013) performed pot culture experiment with maize and revealed that seed bacterization with *Pseudomonas* not only significantly enhanced total dry mass of maize but also increased uptake of Zn, N, K and Mn. Hence fluorescent pseudomonads with Zn solubilizing activity may improve maize growth promotion in more than one ways.

Fluorescent pseudomonads are predominantly known for their ability to produce phytohormones (Spaepen 2011). It has been found that presence of IAA-producing bacteria in rhizosphere has a direct correlation with stimulation of root proliferation (Persello-Cartieaux et al. 2003; Spaepen et al. 2007). It has been found that in the maize rhizosphere auxin producing fluorescent pseudomonads have direct impact on plants health (Picard et al. 2004; Picard and Bosco 2005). In the present study concentration of IAA produced by various isolates varied from 2 to 23 mg l⁻¹. Mutluru and Konada (2007) also reported that IAA production by bacteria could vary among different species and strains, and it is also influenced by conditions, growth stage, and substrate availability. In this study, isolate

PFJM1 produced maximum amount of IAA. In a study Karnwal (2009) screened fluorescent pseudomonads for their ability to produce IAA and found that *P. fluorescens* AK1 gave best results when grown in the presence of L-tryptophan. IAA production in chemically defined media has also been reported in other PGPRs such as *Azotobacter* (Muller et al. 1989), *Azospirillum* (Remans et al. 2008), *Rhizobium* (Hirsch and Fang 1994) and *Bacillus* (Holl et al. 1988) and found to help in plant growth promotion. Wahyudi et al. (2011) also stated that the property of synthesizing IAA is considered as an effective tool for screening beneficial microorganisms. IAA production by fluorescent pseudomonads has been considered as an important attribute to affect plant growth in diverse ways (Somers et al. 2004; Spaepen et al. 2007). For example, in a study Khare and Arora (2011) found that IAA produced by *P. aeruginosa* has also indirectly resulted in suppression of charcoal rot disease of chickpea.

A lot of studies conducted on fluorescent pseudomonads confirmed their versatility in production of many phytohormones (Pallai 2012; Vacheron 2013) and auxins, GAs and cytokinin are the best studied phytohormones (Vacheron 2013; Egamberdieva and Jabborova 2015). The GAs are naturally produced by higher plants, fungi, and bacteria and regulate plant growth and development. In maize GAs influence a range of developmental processes such as stem elongation, germination, grain yield and seed weight (Naghashzadeh et al. 2009). In the present study, GA was produced in the range of 0.13 to 4.3 mg l⁻¹. As in the case of IAA, it was PFJM1 isolate produced maximum amount of GA. Feklistova and Maksimova (2009) reported GA production by *Pseudomonas aurantiaca*. GA producing fluorescent *Pseudomonas* can be used as water stress releaser in maize crops. For example, in a study Kaya et al. (2006) found that application of fluorescent pseudomonads improved the water deficit tolerance in maize seedling by maintaining membrane permeability, enhancing chlorophyll, relative water, and some macronutrient contents in leaves. In a study Abd El-

Ghany (2015) also showed that maize biomass and other growth parameters are influenced in presence of phytohormones secreted by fluorescent pseudomonads.

The requirement of Fe in maize depends on soil conditions. Clark (1982) identified maize as being sensitive to Fe deficiency while Kayode and Agboola (1983) observed that Fe, is essential for large yields. Recently Xue et al. (2014) revealed role of Fe in reproductive stages of maize for increasing yield. Siderophore production in low Fe conditions is an essential trait of fluorescent pseudomonads. Maize cropping systems under low Fe can be improved by application of rhizospheric fluorescent pseudomonads producing siderophore. Several workers reported that under low Fe conditions, fluorescent *Pseudomonas* predominately produced yellow-green fluorescent Fe-binding siderophores (De Silva and Almeida 2006). In the present study, half of the isolates showed siderophore production. The ability of *Pseudomonas* to grow and to produce siderophores is dependent on the Fe content and the type of carbon sources in the medium (Rachid and Ahmad 2005). However, production of different types of siderophores by fluorescent pseudomonads involved utilization of different siderophore-mediated Fe transport systems (Ahmed and Holmström 2014). Dumas et al. (2013) also reported that depending on environmental conditions a single sp. of *Pseudomonas* can produce different siderophores which assure a selective advantage to the plants. Sharma and Johri (2003) showed that use of siderophore producing strains of *Pseudomonas* in maize increased germination percentage, shoot and root length and dry weight. In a study, Shirley (2011) also explained beneficiary effect of siderophores produced by fluorescent pseudomonads on maize. Some workers also reported indirect role of siderophores produced by fluorescent pseudomonads in prevention of plant disease caused by phytopathogen. Role of siderophores produced by pseudomonads was found in controlling the wilt diseases of potato caused by *F. oxysporum* (Schippers et al. 1987) and deficiency diseases of wheat and barley growth caused by *Gaeumannomyces graminis* (Voisard et al.

1989). In maize and peanut they were also found to suppress the phytopathogens (Pal et al. 2001). In present study, PFJM1 isolate was the best siderophore producer and found to produce different types of siderophore which assisted indirectly in maize growth promotion and disease suppression of phytopathogenic fungi.

Various workers examined the role of HCN production by fluorescent pseudomonads as antagonistic factor that contributes to control phytopathogen (Voisard 1989; Paszkowski 1998; Blumer and Haas 2000). In the present study, maximum isolates were found to produce HCN. In a study, Pal et al. (2001) showed that HCN producing fluorescent pseudomonads isolated from maize rhizosphere were found antagonistic to *F. moniliforme*, *F. graminearum* and *M. phaseolina*. In a study, Nadège et al. (2015) also found that amongst different rhizobacteria isolated from maize rhizosphere, the fluorescent pseudomonads were the best HCN producers. In this study PFJM1 also showed HCN production and thought to have its role in showing antagonism against phytopathogens, but further study to explore biocontrol potential of PFJM1 also showed involvement of antifungal compound.

M. phaseolina and *F. moniliforme* are reported as the major soil borne fungi to affect maize plants. Every year soil borne fungi caused severe crop loss. Biocontrol of soil borne fungi by application of fluorescent pseudomonads has been found to be very effective as compared to other microbes (Haas and Défago 2005; Weller 2007). Present study was also aimed to identify fluorescent pseudomonads which were effective antagonist of maize stalk and ear rot pathogens i.e. *M. phaseolina* and *F. moniliforme*. Amongst all, 23% isolates were reported to show *in-vitro* antifungal activity against both phytopathogenic fungi. Isolate PFJM1 showed maximum antagonistic activity (77% reduction in *M. phaseolina* and 69 % of *F. moniliforme*) on dual culture plates. Gupta et al. (1999) also confirmed the antagonistic behavior of the fluorescent pseudomonads isolated from potato rhizosphere against *M. phaseolina* and *F. oxysporum* by dual culture technique and the growth inhibition was found

to be 74.1% and 70.5% respectively. Role of fluorescent pseudomonads in biocontrol of rot diseases is also reported by some workers. Gupta et al. (2002b) reported that charcoal rot of peanut caused by *M. phaseolina* can be suppressed by fluorescent pseudomonads. Govindappa et al. (2011) also showed biocontrol of *M. phaseolina* causing root-rot of safflower and Akhtar and Siddiqui (2009) in chick pea by *P. fluorescens*. In maize *in-vitro* suppression of *F. moniliforme* with strains of *P. cepacia* was reported by Hebbar et al. (1992) and the result indicated 23–80% suppression of seedling infection. Yasmin et al. (2014) evaluated antagonistic behavior of *P. aeruginosa* Z5 isolated from cotton against *F. oxysporum*, *F. moniliforme*, *F. solani* and *Rhizoctonia solani* pathogen and found 25 to 91.5% suppression of growth. Although a lot of work has been done on the antagonistic action of fluorescent pseudomonads against different fungal pathogens yet in the case of maize ear and charcoal rot disease negligible reports are available. In present work, antifungal activity of the isolate PFJM1 against maize ear and charcoal rot-causing fungi were also evaluated by dual culture method.

The potential ability all fluorescent pseudomonads, isolated from different plants, for their PGP activities and to control maize ear rot and charcoal rot fungi were evaluated. It was found that although most of the isolates showed typical PGP traits and varying amount of biocontrol ability against both the fungi but the isolate PFJM1 was the best one. This isolate showed maximum growth reduction of pathogenic fungi *M. phaseolina* and *F. moniliforme*. As the biocontrol of ear and charcoal rot disease was an essential part of this study this isolate was chosen for further study. The isolate was characterized by 16 S rRNA gene sequencing. The 16S rRNA gene from *Pseudomonas* spp. contains 1492 nucleotide positions, of which 148 are variable and 65 positions of these are within three hypervariable regions (Moore 1996). Sequence analysis of the isolate PFJM1 16S rRNA genes showed similarity with 16S rRNA genes of other fluorescent *Pseudomonas* and the isolate (accession number:

KT734728) shared maximum (99%) similarity with fluorescent *Pseudomonas* group (taxid:136843). Neighbor-joining phylogenetic tree clearly demonstrated that the strain belonged to *P. fluorescens*.

It was noticed that biocontrol activities of fluorescent pseudomonads are attributed due to the production of various secondary metabolites having antibacterial, antifungal, antiviral and antinematicidal activity (Haas and Defago 2005). In this study, PFJM1 showed *in vitro* antifungal activity against phytopathogenic fungi. Isolate PFJM1 was found to produce antifungal metabolites, HCN, and siderophores that contributed in inhibition of pathogen growth, suggesting its potential as biocontrol agent of plant diseases. Further it was found that isolate produced antifungal metabolites as active compounds responsible for biocontrol activity against both the phytopathogenic fungi *M. phaseolina* and *F. moniliforme*. Solvent extraction and TLC of the compound showed two fractions. One fraction of the crude compound showed antifungal activity against both the fungi and its Rf value was 0.78. Previous studies conducted for exploring antifungal compounds secreted by fluorescent pseudomonads showed that solvent extracted crude metabolites show different Rf values on TLC plate (Reddy and Rao 2009). According to Rosales (1995) under UV light blue color of pyocyanine showed at Rf of 0.51 to 0.53 and pyrrolnitrin at Rf of 0.23 to 0.28. However, bright yellow spots at Rf value from 0.35 to 0.88 are reported for 2,4- Diacetyl phloroglucinol (DAPG) (Rosales 1995; Kavitha et al. 2004; Showkat et al. 2012). Partial characterization by UV-Visible and FT-IR spectrophotometry also showed the similarity of the compound with 2,4-DAPG. UV-Visible spectrum demonstrated a nearly identical λ max (270 nm) of this peak with 2,4-DAPG. FTIR spectrum showed (cm^{-1}) 3400-3200 (OH br) 1600-1400 (aromatic conjugated C=O with intramolecular hydrogen bonds and aromatic C=C), 1200–1000 (COH) are similar to synthetic DAPG (Marchand et al. 2000). These spectroscopic data concluded that the second fraction of compound belong to DAPG. It has been also observed

in some studies that in multiple biocontrol properties, production of non-volatile metabolites by fluorescent pseudomonads have maximum effect on fungal growth suppression (Frapolli et al., 2007; Asadhi et al. 2013). In the present study, we found that DAPG directly inhibited the growth of *M. phaseolina* and *F. moniliforme* when bioassay of TLC fractionated purified compound was performed by agar well method. In a study, Mishra and Arora (2012) also found that DAPG producing fluorescent pseudomonads had the potential to suppress black rot fungi in cabbage.

DAPG produced by strains of *P. fluorescens* is a major metabolite involved in the biological control of several plant diseases. Earlier work demonstrated the role of DAPG in the suppression of black root rot of tobacco (*Thielaviopsis basicola*), take-all of wheat (*Gaeumannomyces graminis* var. *tritici*) (Keel et al. 1992; Mazzola et al. 1995; Weller 2007), damping-off of sugar beet (*Pythium ultimum*) (Fenton et al. 1992; Shanahan et al. 1992) and Fusarium wilt (McSpadden Gardener et al. 2000)). Andres et al. (2011) also reported the antifungal activity of DAPG against phytopathogen *M. phaseolina*. Landa et al. (2002) isolated DAPG-producing *P. fluorescens* from roots of pea plants and indicated that these bacteria were involved in suppression of Fusarium wilt disease (*F. oxysporum* f.sp. *pisi*). Manikandan et al. (2010) and Selvaraj et al. (2014) noticed that DAPG producing *P. fluorescens* reduced *F. oxysporum* infection in chickpea. DAPG producing fluorescent pseudomonads also provide biocontrol of many other root and seedling diseases in a variety of crops (Défago 1993). Although DAPG is produced by pseudomonads of worldwide origin, its biosynthetic locus is conserved in pseudomonads obtained from diverse geographic locations (Keel et al. 1996; Raaijmakers et al. 1997). However, only a limited number of attempts to characterize maize rhizospheric fluorescent pseudomonads for DAPG production and examine their role in controlling maize rot diseases has been carried out. Earlier, Piccard et al. (2000) studied frequency and biodiversity of DAPG producing *Pseudomonas* isolated

from the maize rhizosphere at different stages of plant growth, and they found that 188 of the total 1,716 isolates were able to produce DAPG at different stages of growth. In present study, PFJM1 is characterized as DAPG producing bacterium from maize rhizosphere and its role in suppression of ear rot and charcoal rot of fungi was confirmed. Characterization and revelation of morphological abnormalities such as perforation, fragmentation, swelling, shriveling and lysis of hyphae of pathogenic fungi by metabolite produced by fluorescent pseudomonads further strengthens our understanding of the interaction of the bacterium with fungal pathogen. In this study, SEM observation of *M. phaseolina* taken from intersection area with the zone of inhibition revealed bending of mycelium, fragmentation, absence of sclerotia formation and other structural deformities. However, in *F. moniliforme* both mycelial and conidial deformities were observed.

As reported by other researchers, the fluorescent pseudomonads have been considered as main bacterial genera capable of showing multiple PGP and biocontrol activities (Rana et al. 2011). Several workers reported that rhizospheric bacteria showing more than one trait of PGP are known as multi-trait PGPR (Rana et al. (2011; Almario et al. 2014; Tewari and Arora 2015). The present study, therefore, also supported the view of fluorescent pseudomonads as a multi-trait PGPR. Although numerous studies have been postulated and demonstrated PGP and biocontrol activity of *P. fluorescens* but their use in the form of bioinoculant can be best assessed in plants and field conditions. Hence, the pot experiment was also conducted to see the effect of PFJM1 on maize growth and disease suppression by phytopathogenic fungi. In the pot study, seed treated with PFJM1 reduced ear and charcoal rot disease by 68 and 74% respectively in comparison to pathogen control. There are several instances where seed biopriming with different strains of fluorescent pseudomonads decreased the disease severity in several crops. For example, in a study, Ardakani et al. (2010) confirmed 82 % reduction in cotton seedling damping-off caused by *R. solani* by

application of *P. fluorescens* compared to the control. Das et al. (2008) found in a pot experiment that *P. chlororaphis* SRB 127 isolated from sorghum rhizosphere showed strong antagonistic effect against charcoal rot pathogen of peanut and sorghum (*M. phaseolina*). In the present study, seed bacterization with PFJM1 also increased germination percentage in comparison to control. This was probably due to the effective colonization of PFJM1 on seed and secretion of phytohormones and antifungal metabolites against both the fungi. PFJM1 was found to be most effective in enhancing plant growth parameters even 90 DAS. PFJM1 bioformulation also showed significant increase ear fresh weight, dry weight, number of seed per plant and 100-grain weight in comparison to control. Use of fluorescent pseudomonads for growth enhancement in other than maize is also reported. For example, in a study, Goswami et al. (2013) observed treatment of fluorescent pseudomonads enhanced overall fresh biomass by 24% and overall dry biomass by 27% of chickpea after 15 days of seeded in pots. Similarly, Jarak et al. (2012) also reported that inoculation of fluorescent pseudomonads enhanced maize growth in greenhouse and field conditions.

It has been noticed that some bacteria sharing good PGP activities in planta condition may lose or show low level of PGP activities during field application (Hol et al. 2013; Pereg and McMillan 2015). Research in this area is still confined to the laboratory and very little attention has been paid to produce the bioformulations of the effective strains for field applications. Hence in the present crop production scenario, field application of PGPR in the form of suitable bioformulation is of utmost importance (Burgess and Jones 1998). For bioformulations selection of appropriate carrier material which must offer special characteristics such as high water-holding capacity, chemical and physical uniformity, lack of compounds toxic to microbial strains and be environmentally safe is crucial (Albareda et al. 2008). According to Stephens and Rask (2000) these materials should have near neutral or readily adjustable pH and be abundant locally at a reasonable cost (Ferreira and Castro 2005).

In the present study, talc was used as an inorganic carrier which fulfills the criterion mentioned above. In a study by Sharma et al. (2011) inorganic carrier, talc powder was used for preparing the bioinoculant formulations of fluorescent pseudomonads was found very effective in growth promotion and disease resistance activities for tomato plants. Various workers also described advantages of talc as carrier in bioformulation (Vidhyasekaran et al. 1997; Bashan 1998; Bora et al. 2004; Sallam et al. 2013; Tewari and Arora 2014). According to Beatty and Jensen (2002), a successful biocontrol agent must survive in formulation and storage and must be a competitive and aggressive colonizer after inoculation. The survival of PFJM1 was checked in bioformulation till six months of storage and it was found that its population density was sufficient to colonized maize plant. In a study, Suryadi et al. (2013) also reported that the cell viability of *Pseudomonas* remained more stable in talc based formulation. Rajalaxmi et al. (2012) reported talc based formulations of *P. fluorescens* retains mean population of 11×10^7 cfu/g 300 days of storage.

Field trials were done at two sites with diverse soil properties and pathogen presence. According to Thieseen and Woodward (2012), soil physicochemical properties influences the severity of infection caused by soil-borne fungi. The soil of greenhouse field of BBA University is alkaline and nutrient poor. The soil is also reported to be infested with *M. phaseolina* (Tewari and Arora 2015). However, soil of the Itaunja village (Sitapur, UP, Lucknow) was only slightly alkaline and fertile. Farmers have reported prevalence of ear rot of maize in this region. Seed treatment with fungicides is a practice that has been used by an increasing number of farmers growing cereal crops. Thiram is the most common synthetic fungicide used before sowing by local farmers to treat the seed-borne fungal diseases of maize. In a study, Tonin and Avozani (2013) studied *in vitro* mycelial sensitivity of *M. phaseolina* to various fungicides including Thiram and found that higher concentration of active ingredient is required to treat fungus. So apart from untreated seed control and PFJM1

coated seeds, a Thiram treated seed treatment were also included in both the fields to compare its effect against PFJM1 treatment.

In both fields studies, PFJM1 showed its effectiveness in controlling ear rot and charcoal rot incidence and was even better in comparison to Thiram treated seeds. Reduction of disease incidence could be due to siderophore production and secretion of HCN and antifungal metabolites in rhizosphere. Apart from this better survival of PFJM1 in maize rhizosphere decreased the deleterious effects of phytopathogenic fungi. Many studies also analyzed the impact and the survival and ability of fluorescent pseudomonads in maize rhizosphere. According to Botelho et al. (1998), *P. fluorescens* inoculated with maize had maintained their number and showed better survival over other competing microbes. Guimarães et al. (1997) also observed that strain of *P. fluorescens* adequately survived and/or colonized in bulk soil and rhizosphere of maize. In this study, it was also found that in both the field experiments seed coated with PFJM1 enhanced in maize growth parameters over control. Itanunja field crop harvested 90 DAS showed a fairly increase plant growth parameters over control. Yield in terms of ear fresh weight, ear dry weight, number of seed per plant and 100 grain weight was also found to increase. In field, harvest loss due to ear rot fungus is already reported by local farmers and use of PFJM1 bioformulation proved very successful in attaining the plant to acquire healthy silking stage (most susceptible for *F. moniliforme* attack). In a similar study Viruel et al. (2014) showed a positive effect of fluorescent pseudomonads on maize plant growth. They pointed that inoculation of maize with *P. tolaasii* IEXb increased plant height (45%), shoot dry weight (40%) and *P. koreensis* SP28A increased seedling emergence (8%), shoot length (19%), grain yield (44%), 1000-grain weight (18%), total dry biomass (32%) and P content (56%) of maize plants. Similar studies where application of PGPR improved plant growth parameters in other cereals such

as sorghum (Raju et al. 1999), pearl millet (Niranjan et al. 2004), wheat (Shaukat et al. 2006a) and sunflower (Shaukat et al. 2006b) have been reported.

Results obtained in field trial at BBA University were also quite similar to the Itaunja village. As the field was infested with *M. phaseolina* and in presence of fungi (negative control) plant showed clear and classical disease symptoms. But when PFJM1 was applied the disease incidence was reduced significantly. However, in the presence of PFJM1 bioformulation, not only the overall growth parameters of maize were enhanced, but maize yield also increased. This was probably due to combined action of PGP and biocontrol traits of PFJM1 in maize rhizosphere. Contribution of root colonizing DAPG-producing pseudomonads and their ability to suppress pathogen activity in the rhizosphere of wheat were also reported by Raaijmakers and Weller (2001). Plant growth parameters were enhanced in the presence of PFJM1 as the isolate was a potent IAA, GA and phosphate and Zn solubilizer. In a study, Zafar-ul-Hye et al. (2015) studied effect of *Pseudomonas* for promoting root and shoot growth of maize. The data revealed that in presence of *Pseudomonas* root and shoot length, root and shoot fresh weight, and root and shoot dry weight increased up to 34, 43, 35, 71, 55 and 68%, respectively. Studies also indicate that bioinoculants containing *Pseudomonas* in consortia also worked well. For example, a study conducted by Umesha et al. (2014) evaluated efficiency of biofertilizers (containing *Pseudomonas* in consortia) on growth and yield of maize. Results of their study indicated that treatment with *P. fluorescens* in consortia showed highest plant height, total dry matter, weight of cob and grain yield per plant.

Agriculture is under increasing pressure to reduce the use of synthetic chemical pesticides for controlling the phytopathogens, because of concerns about the adverse impact of pesticides on public health and the environment. Thus, there is considerable interest in finding and developing bio-based approaches such as bioformulation products with disease-

eradicating potential against phytopathogenic fungi. In this regard use of rhizospheric microbes was proven beneficial. According to Figueroa-López (2016), co-existence of native soil microbiota in the crop for many years confer competitive advantages compared to exotic species. The research presented here was also aimed to identify best PGP and antagonist fluorescent pseudomonads for developing a suitable bioformulation that could provide a valuable impact on maize crop in diverse field conditions. According to Jha et al. (2010) and Arora et al. (2016), easily cultivable microorganisms can be efficiently utilized in developing bio-fertilizers/bioformulations against crops diseases. Findings in this study with PFJM1 suggests it as a potent PGP and biocontrol bacterium as evident from *in-vitro*, in planta and field applications. In the present study efficacy of the PFJM1 isolate in the form of bioformulation also suggests its exploitation as an alternative to mineral fertilizers for widespread use in maize crop sown in the various geographical region of India.

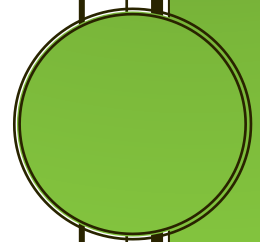
A lot of studies show fluorescent pseudomonads as producer of diverse antifungal compounds and exhibiting multiple PGP traits. But in cereals like there are very few reports on controlling phytopathogen of maize and simultaneously enhancing the yield. Strain *P. fluorescens* PFJM1 not only enhanced plant growth but was also able to suppress diseases by controlling phytopathogens *M. phaseolina* and *F. moniliforme*. More specifically, the study used a combination of PGP assays, in planta and field experiment to examine how in the presence of fluorescent pseudomonads rot disease incidences can be minimized. The study also suggested that selection and use of DAPG producing fluorescent pseudomonads from maize rhizosphere can exert marked effects on the prevalence and abundance of soil borne fungal pathogen of maize.

Recently Vacheron et al. (2016) showed that fluorescent *Pseudomonas* strains with only a few plant-beneficial properties are favored in the maize rhizosphere. Although this is contrary to the general prediction that a given PGPR strain displaying several different plant-

beneficial properties (Loper et al. 2012), will provide higher positive effects on the plant (Bashan and de-Bashan 2010). However, finding as given by Vacheron et al. (2016) unravels the intricate behavioral association of fluorescent pseudomonads residing with maize rhizosphere. Furthermore, the discovery of such isolates may be considered as potential source of novel approaches to treat maize diseases and increase productivity at a wider scale. Similarly, a more extensive research on various antifungal bioactive metabolites produced by fluorescent pseudomonads will provide them an iconic recognition amongst all biocontrol bacterium to develop new biocontrol agents for controlling diverse pathogenic fungi in agriculture. In this regard, using their metabolites in the development of bioformulation could also be significantly impacted in sustainable agriculture (Mishra and Arora 2016).

In India, maize is the third most important food crop after rice and wheat. The crop is used as staple food by human and quality feed for animals. Apart from this, maize also serves as a basic raw material as an ingredient to thousands of industrial products. In spite of several technological advances, its production and yield is severely affected by various soil borne diseases. Further rot diseases caused by soil borne fungi also found to deteriorate seed quantity and quality. Even the most practical and economical way of controlling the rot diseases which are development of resistant cultivars, crop rotation and treatment with fungicides were found to be inefficient in enhancing maize yield. However, use of bioformulation has emerged as an attractive and ecofriendly approach to treat diseases and enhance productivity in agroecosystem. Use of multi-talented strain such as PFJM1 reported in this study can be important to achieve the target of food security in a sustainable and eco-friendly manner. A tailor-made formulation for a very important group (maize) can be very useful in providing chemical free and higher yield from ever shrinking agricultural lands.

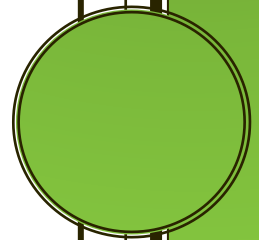
CONCLUSION



7. CONCLUSION

The work of present study concludes that among various isolates, PFJM1 not only showed the potential of enhancing maize growth but also inhibited ear and charcoal rot pathogens. Application in pot and field study suggests that bioformulation of fluorescent *Pseudomonas* can be used as an alternative to mineral fertilizers for increasing the productivity of maize crop. Similarly, eradication of maize soil-borne phytopathogens by the application of *P. fluorescence* JM-1 will provide a cheaper and safer alternative to chemical fungicides. Determination of active metabolites responsible for selective toxicity to these phytopathogens could also assist to bioformulations market, and especial bioformulation of biopesticides can be prepared for geographical region where ear and charcoal rot diseases are more common and can't be treated by sole application of synthetic fungicides.

SUMMARY



8. SUMMARY

Cereal crops have always been the main source of calorie in our diet. Cereals like wheat, rice and maize are the most important food commodities. In India, maize is the third most important food crop after rice and wheat and contributes a lot in daily protein requirement. In last few years production of maize has substantially increased at global level but this rise in production also emphasized overuse of mineral fertilizers in maize fields. Similarly, it has been also observed that measures applied in maize disease control are inefficient and involved harsh chemicals/pesticides. Rot diseases of maize have global significance and amongst them ear and charcoal rot are considered most destructive. In India, estimated losses due to major diseases of maize is about 13.2% of which 5 % is caused by rot diseases which results in heavy yield loss. As already mentioned, in absence of effective measures of maize disease eradication every year a percent of yield loss is increasing. Hence there is an urgent need to formulate biological approaches to treat maize diseases and enhance productivity in sustainable manner. Rhizospheric microbes have are known as suitable candidates for enhancing plant growth. Amongst the rhizospheric microbes, fluorescent pseudomonads are considered as dynamic PGPR, influencing plant health by several activities.

In the present study 42 fluorescent pseudomonads were isolated from diverse crops grown in Lucknow and nearby regions. Further isolates were characterized on the basis of morphological and biochemical characteristics which showed isolates, resemblance with fluorescent pseudomonads. All the isolates were also monitored for their PGP and biocontrol activities against ear and charcoal rot fungi (*F. moniliforme* and *M. phaseolina*). On the basis of various PGP traits and biocontrol potential against rot fungi, the isolate PFJM1 was found to be most efficient among all and hence was further characterized by 16S rRNA gene

sequencing. 16S rRNA genes of isolate PFJM1 (accession number: KT734728) shared maximum (99%) similarity with fluorescent *Pseudomonas* group (taxid:136843).

A comprehensive study of all the isolates for monitoring their PGP and biocontrol activities were also conducted. The study confirmed that maximum isolates were able to solubilize P, Zn and produce phytohormones (IAA and Gas). Apart from this, siderophore, HCN production and biocontrol activity against *M. phaseolina* and *F. moniliforme* was found to be present in some isolates. Amongst all, PFJM1 displayed maximum PGP traits and also showed biocontrol potential against rot fungi and was selected for further study.

Role of PFJM1 in suppression of rot fungi i.e. *M. phaseolina* and *F. moniliforme*. was also observed under SEM. In SEM bending of mycelium, fragmentation, absence of sclerotia formation and conidial deformities were observed. Isolate PFJM1 was found to produce HCN and different types of siderophores that can contribute to inhibition of pathogenic fungi. The initial separation of crude metabolite of the strain *P. fluorescens* JM-1 by using TLC revealed two major spots with Rf values of 0.74 and 0.78. Of which one spot (Rf 0.78) showed antifungal activity against both the fungi as evident in bioassay. Partial characterization by UV-Visible and FT-IR spectrophotometry showed the similarity of this compound with 2,4-DAPG. UV-Visible spectrum demonstrated a nearly identical λ max (270 nm) whereas FTIR spectrum showed (cm⁻¹) 3400-3200 (OH Br) 1600-1400 (aromatic conjugated C=O with intramolecular hydrogen bonds and aromatic C=C), 1200–1000 (COH), similar to synthetic DAPG.

Hence isolate PFJM1 showed production of various metabolites including DAPG. considered as multi-trait PGP and DAPG producer. Effect of PFJM1 on maize growth enhancement and rot diseases suppression were also monitored in pot and field experiments. In pot experiment seed treated with PFJM1 showed reduced ear and charcoal rot disease (by 68 and 74% respectively) in comparison to pathogen control. Apart from disease their was

enhancement in reduction maize growth parameters. Results of pot experiment clearly indicated usefulness of PFJM1 as potential bioinoculant for maize growth enhancement and disease suppression of rot fungi.

To prove efficacy of PFJM1 in treating rot diseases and growth enhancement in field conditions, a talc based bioformulation was also prepared. The survival of PFJM1 was checked in bioformulation till six months of storage and it was found that its population density was maintained above 1×10^8 CFU g^{-1} . The bioformulation was applied in two different fields, where incidence and prevalence of rot diseases were already reported. The soil of BBA University is alkaline and nutrient poor and reported to be infested with *M. phaseolina*. Here application of PFJM1 bioformulation reduced disease incidence. However, soil of the Itaunja village field (Sitapur, UP, Lucknow) which was slightly alkaline and fertile showed 78% reduction in disease incidence (of *F. moniliforme*) when treated with PFJM1 bioformulation. At Itaunja prevalence of ear rot of maize is reported by local farmers and synthetic fungicide Thiram is being used by local farmers to treat the seed-borne fungal diseases of maize. However, result of both fields studies indicated that use of PFJM1 was found very effective in controlling ear rot and charcoal rot incidence in comparison to untreated and Thiram treated seeds. Apart from this, seed coated with PFJM1 were also found to enhance maize growth parameters over control. Yield in terms of ear fresh weight, ear dry weight, number of seed per plant and 100 grain weight was also found to increase sign of deficiency 90 DAS (over control) in both the fields.

In brief, from present study it can be stated that use of multi-trait PGP and biocontrol metabolites producing *P. fluorescens* not only enhanced maize growth but also effectively suppressed rot diseases in maize caused by soil borne fungi. The study also reports development of effective talc based bioformulation from DAPG producing *P. fluorescens* JM-1 development. The issues related to maize yield in terms of quantity and quality can be

resolved by application of such multi-trait strain leading to food security in a sustainable manner.

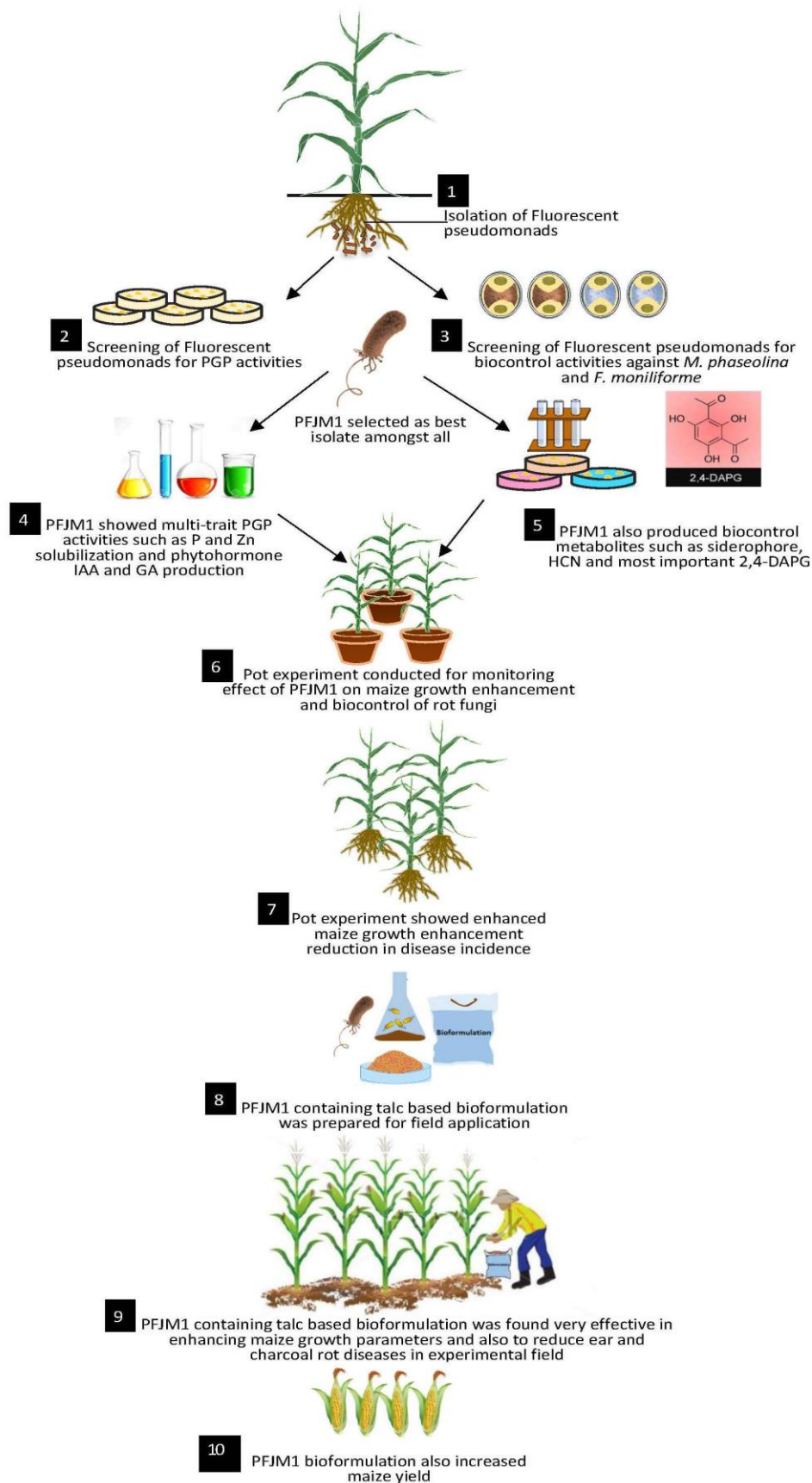
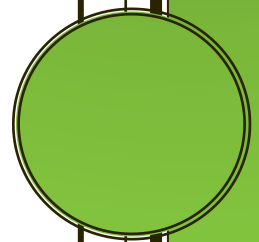


Figure 51. Summary of the work done

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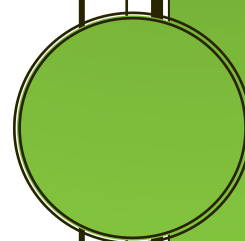
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PUBLICATIONS



10. PUBLICATIONS

Research Papers

Bansal OP, Mishra J (2012) An estimation of microbial count including nitrogen Fixing bacteria in agricultural fields of Aligarh district Irrigated with untreated sewage water. *Inter J Nat Sci* 3(2):259-262

Mishra J, Rajnandani M, Arora NK (2016) Biocontrol of ear rot fungi by plant growth promoting fluorescent pseudomonads. *Inter J Sci Technol Soc* 1(2):49-57

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Mishra J, Tewari S, Singh S, Arora NK (2015) *Biopesticides: Where We Stand?* In: Arora NK (ed). *Plant Microbes Symbiosis: Applied Facets*: Springer India, pp 37-75

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AN ESTIMATION OF MICROBIAL COUNT INCLUDING NITROGEN FIXING BACTERIA IN AGRICULTURAL FIELDS OF ALIGARH DISTRICT IRRIGATED WITH UNTREATED SEWAGE WATER

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ABSTRACT

In the peri-urban areas of India sewage water is a valuable resource for agricultural production. This study pertains to the investigation of the impact of domestic and industrial sewage water irrigation on chemical and biological characteristics in alluvial soils of Aligarh district. Ten fields were selected which were irrigated by only sewage water (SI), eight selected fields were partially irrigated by sewage water (PSI) and for the three additional selected fields the source of irrigation was tube well water. The results denoted that the soil organic matter and CEC increased with sewage irrigation and duration of irrigation. The data of study indicated that there was a significant increase in the bacterial and fungal population and decrease in actinomycetes population in sewage irrigated soils as compared to control. The population density of bacteria and fungi in sewage irrigated soils increased with the duration of sewage irrigation. Soil organic matter was significantly positively correlated with soil bacterial and fungal population, and negatively correlated with actinomycetes. The results also showed that the number of a symbiotic nitrogen fixing bacteria decreased in presence of sewage water, denoting that mineralization of nitrogen decreased in presence of sewage water, while sewage water had no appreciable effect on sulfur-oxidizing bacteria. The total nitrogen content and total heavy metals concentration in SI were 2-2.4 times than control, while in PSI it was 1.5-1.6 times. The concentration of DTPA extractable heavy metals showed no difference between partial sewage irrigated and sewage irrigated soils, which may be due to deposition of heavy metals in crops grown on the soils.

KEY WORDS: sewage irrigation, alluvial soil, Microbial population, heavy metals.

INTRODUCTION

Maintenance of good soil quality is of prime importance for sustainable agriculture. Heavy metals constantly interplay with human life environmentally, industrially and biologically (Doran and Zeiss, 2000). The toxicity resulting from the continuous and excessive exposure of heavy metals poses a grave risk factor to human health. Accumulation of toxic metals in human beings causes toxic effects like inhibition of haemoglobin formation, sterility, hypertension, kidney damage and mental retardation (Muller *et al.*, 2007). In India, there is a gradual decline in freshwater availability for agricultural fields so sewage and other industrial effluents are being used for irrigation of agricultural fields particularly in periurban areas. The indiscriminate disposal of industrial and sewage effluents on agricultural lands is becoming a major source of heavy metal contamination in irrigated soils and ground water (Elgala *et al.*, 2003; Patel *et al.*, 2004). The uptake of heavy metals from contaminated soils by plants comprises a major path for such elements to enter the human and animal food chain (Ghafoor *et al.*, 2004). Plant uptake of metals from sewage treated soils is related to the soil exchange capacity, pH of soil as well as the sewage contamination (Adhikari and Gupta, 2002). Soil biology is a significant component of soil quality and microorganisms play vital roles in soil fertility and primary production through organic matter decomposition and nutrient cycling. When some stress factors such as

temperature, extreme pH or chemical pollution are imposed on a natural environment, soil biota can be affected as well as these micro-organisms regulate the ecological processes. In general, an increase of metal concentration adversely affects soil microbial properties *e.g.* respiration rate, enzyme activity, which appears to be very useful indicators of soil pollutions (Pawloska and Charvat, 2004). Microorganisms including bacteria, fungi, actinomycetes serve as indicator for studying the harmful effects of metals at the cellular level. These microorganisms can also be used to remove toxic metals from contaminated sites as they can efficiently accumulate heavy metals and radionuclides from their external environment (Ali and Wainwright, 1995). Present study enumerates of general soil microbes as well as some specific microbes including sulfur oxidizing, nitrogen fixing of different soils of Aligarh irrigated by sewage water/ partially irrigated by sewage water.

MATERIALS AND METHODS

Soil samples (0-25 cm) from ten sewage irrigated, eight partially sewage irrigated and three ground water irrigated were collected bimonthly from different agricultural lands of Aligarh districts (27°53'N 78°35'E) (from each site 5-7 samples were collected and were bulked) from the month of June 2010. The collected soil samples were brought to the laboratory in sterile polythene bags and stored at 4°C for further analysis.

Degumming of silk filaments spun under varied climatic conditions

The collected soil samples were air-dried, grounded and sieved through 2 mm sieve. The soil characteristics like soil moisture, pH, electric conductivity, organic matter, nitrogen and phosphorous were determined by usual methods (Bansal, 1982). Total and DTPA extractable heavy metals namely Cu, Cd, Zn, Cr, Pb and Ni were determined by atomic absorption Spectrophotometer (Lindsay and Norvell, 1978). The results are given in Tables 1 and 2.

Isolation of bacteria, fungus, Actinomycetes was made in each soil sample using dilution plate techniques; dilution was up to 10^{-7} for bacteria, 10^{-5} for fungus and 10^{-3} for actinomycetes (Cuppucino and Sherman, 1983) The results are given in Table 3.

Asymbiotic nitrogen fixing bacteria were estimated as: Nitrogen-Free Glucose broth medium was used for enrichment and nitrogen-Free Glucose agar (Benson) for isolation. Medium contains 1.0 g of K_2HPO_4 , 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$ and 0.001 g of $Na_2MoO_4 \cdot 2H_2O L^{-1}$ and Glucose solution (10.0 g glucose dissolved in 100 mL water) pH was adjusted to 7.2. Both solutions were sterilized separately at $121^\circ C$ at 15 lbs pressure for 15 minutes. Enrichment cultivation was carried out with 250-mL Erlenmeyer flask containing 10 g of fresh soil and 100 mL of Nitrogen-Free Glucose broth medium for seven days at $30^\circ C$ under oxic conditions without shaking to avoid

disruption of biofilms. Now a loopful biofilm samples were homogenized and diluted up to 10^{-7} and 0.1 ml of the diluted samples were spread over the agar plates of the same medium containing agar and incubated at $30^\circ C$ for a few (6-7) days. The results are given in Table 3.

MPN counts of sulfur-oxidizing bacteria: The MPN technique (Alexander, 1982) was used to estimate the abundance of sulfur-oxidizing bacteria (SOB) in the different samples. For enrichment 10 g of fresh soil was added in 100 mL of thiosulfate mineral salts medium (thiosulfate MSM) and incubated for 20 days at $30^\circ C$ in dark conditions. The medium composition in 1L of distilled water is 2.0 g KNO_3 , 1.0 g NH_4Cl , 2.0 g KH_2PO_4 , 2.0 g $NaHCO_3$, 0.8 g $MgSO_4 \cdot 7H_2O$, 5.0 g $Na_2S_2O_3 \cdot 5H_2O$ and 1.0 mL trace element solution with the pH adjusted to 6 with 1N KOH. The trace element solution contained in 1L of distilled water: 50 g Na_2-EDTA , 7.34 g $CaCl_2 \cdot 2H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 2.5 g $MnCl_2 \cdot 4H_2O$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 g $CaSO_4 \cdot 5H_2O$ and 11.0 g NaOH. After incubation samples were serially diluted to appropriate range for SOB. Between every dilution step, the samples were vigorously shaken on a vortex apparatus to dislodge the bacteria from the sediment particles. From each dilution, five replicate tubes containing growth medium were inoculated and incubated for 4 weeks. Growth was determined by pH reduction test (pH 6 to 3.5 pH reductions). The results are given in Table 3.

TABLE 1. Physicochemical properties of collected soil samples (June, 2010 to April, 2011)

S.N.	Name of sample sites	pH	Organic Matter (%)	CEC Cmol kg^{-1}	Total Nitrogen (%)	Total Phosphorous (%)
	TAPPAL (PSI)	8.23 (8.15-8.32)	2.91(2.84-2.96)	12.5	0.13 (0.12-0.14)	0.052(0.05-0.054)
2	KHAIR (SI)	7.82 (7.7-7.9)	4.26(4.11-4.35)	15.2	0.16 (0.15-0.175)	0.054(0.052-0.056)
3	LODHA (SI)	7.83 (7.75-7.92)	3.88(3.80-3.98)	14.1	0.164 (.155-0.172)	0.050(0.049-0.052)
4	MANDRAK (PSI)	7.93 (7.84- 8.12)	3.03(2.97-3.08)	12.7	0.128 (0.122-0.136)	0.055(0.053-0.057)
5	IGLAS (PSI)	7.81 (7.75-7.91)	2.63(2.5-2.70)	11.8	0.134 (0.128-0.140)	0.052(0.05-0.054)
6	SASANI (PSI)	7.75 (7.74-7.82)	2.95(2.88-3.04)	12.9	0.126 (0.122-0.30)	0.048 (0.047-0.050)
7	GONDA (SI)	7.88 (7.82-8.04)	4.27(4.11-4.42)	15.8	0.158 (0.152-0.166)	0.051(0.05-0.053)
8	KHAIRYA (SI)	8.08 (8.04-8.22)	4.44(4.20-4.64)	16.1	0.154 (0.150-0.160)	0.056 (0.054-0.059)
9	MULLAPADA (SI)	7.96 (7.84- 8.15)	5.16(4.91-5.30)	16.4	0.164 (0.162-0.174)	0.050 (0.049-0.052)
10	DHANIPUR (PSI)	7.84 (7.75-7.94)	3.06(2.98-3.14)	13.0	0.132 (0.128-0.136)	0.048 (0.047-0.050)
11	GANGARI (SI)	8.06 (7.95-8.24)	3.64(3.56-3.72)	14.5	0.164 (0.160-0.172)	0.052(0.05-0.055)
12	KASHIMPUR (G)	8.58 (8.55-8.64)	2.02 (2.0-2.05)	10.2	0.072 (0.070-0.074)	0.032 (0.030-0.035)
13	ATROULI (G)	8.40 (8.35-8.45)	2.11(2.06-2.18)	9.8	0.076 (0.074-0.080)	0.034 (0.032-0.038)
14	KALINADI-1 ST (SI)	7.80 (7.72- 7.90)	5.25(5.12-5.34)	16.4	0.168 (0.162-0.174)	0.058 (0.056-0.062)
15	KALINADI-2 ND (SI)	7.52 (7.45-7.62)	4.62(4.51-4.73)	15.7	0.158 (0.154-0.168)	0.056 (0.054-0.0590)
16	JALALI (PSI)	8.12(8.05-8.22)	3.00(2.94-3.08)	14.0	0.132 (0.128-0.136)	0.048 (0.046-0.051)
17	AKRABAD (PSI)	7.79(7.72-7.94)	2.84(2.80-2.90)	13.4	0.136 (0.132-0.140)	0.049 (0.046-0.053)
18	GABHANA (PSI)	7.52(7.42-7.60)	3.04 (3.0-3.10)	13.0	0.126 (0.124-0.132)	0.052 (0.050-0.055)
19	KILA (SI)	8.36(8.25-8.52)	5.62(5.42-5.82)	16.8	0.166 (0.162-0.172)	0.062 (0.058-0.064)
20	CHANDAUS (SI)	7.58 (7.52-7.70)	3.06(3.03-3.12)	13.0	0.156 (0.152-0.164)	0.051 (0.050-0.051)
21	PANATHI (G)	8.28 (8.25-8.32)	1.82(1.78-1.86)	10.1	0.078 (0.074-0.080)	0.028 (0.027-0.0300)

SI = Sewage irrigated soil; PSI = Partial sewage irrigated soil; G =Ground water irrigated soil

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TABLE 2. Concentration (mg kg⁻¹) of heavy metals in collected soil samples of Aligarh district (June10-April, 11)

S.N.		Zn		Cu		Cr		Pb		Cd		Ni	
		T	E	T	E	T	E	T	E	T	E	T	E
1	TAPPAL (PSI)	68(62-74)	44 (42-48)	29 (27-32)	12 (11-13)	48 (45-54)	28 (26-30)	40 (38-44)	21 (20-23)	0.34 (0.32-0.36)	0.20(0.19-0.22)	16 (14-18)	9.5 (9-11)
2	KHAIR (SI)	97 (94-104)	48 (44-52)	37(35-40)	22 (20-25)	62 (57-64)	34 (32-38)	54 (52-58)	24 (22-26)	0.42 (0.40-44)	0.24 (0.22-0.26)	21 (20-24)	11 (10-13)
3	LODHA (SI)	95 (92-100)	47 (45-49)	34 (32-39)	21 (20-24)	60 (59-62)	32 (30-35)	55 (52-58)	24 (22-27)	0.40 (0.38-0.42)	0.23 (0.21-0.25)	20 (19-22)	11 (10-12)
4	MANDRAK (PSI)	71 (68-75)	46 (43-48)	27 (25-30)	12.5 (12-13.5)	46 (44-49)	30 (28-33)	38 (36-40)	20 (19-22)	0.31 (0.30-0.33)	0.20 (0.18-0.22)	15 (14-17)	9 (8.5-10.5)
5	IGLAS (PSI)	64 (61-69)	40 (38-41)	25 (23- 27)	14 (13-16)	45 (44-48)	29 (28-31)	39 (36-41)	22 (20-25)	0.30 (0.28-0.32)	0.19 (0.18-0.20)	15 (13-17)	9 (8-10)
6	SASANI (PSI)	60 (56-64)	39 (37-41)	24 (23-26)	12 (11-13)	44 (42-48)	29 (28-31)	36 (33-38)	21 (20-23)	0.32 (0.30-0.35)	0.19 (0.18-0.21)	16 (15-18)	10 (9-11)
7	GONDA (SI)	102 (96-110)	51 (48-54)	38 (36-41)	24 (23-27)	64 (62-68)	37 (35-39)	58 (54-62)	25 (23-28)	0.44 (0.42-0.48)	0.25 (0.23-0.28)	22 (20-25)	12.5 (11.5-14)
8	KHAIYA (SI)	96 (92-102)	49 (45-52)	37 (35-40)	23 (22-26)	61 (58-63)	35 (33-38)	55 (52-59)	24 (22-26)	0.42 (0.40-0.44)	0.24 (0.23-0.27)	21.5 (20-23)	12 (11-13)
9	MULLAPADA (SI)	92 (90-96)	47 (45-50)	36 (34-38)	21 (20-23)	64 (62-68)	37 (35-40)	52 (50-55)	25 (23-28)	0.45 (0.42-0.48)	0.25 (0.23-0.27)	23 (22-26)	13 (11-14)
10	DHANIPUR (PSI)	72 (70-78)	38 (36-41)	25 (23-28)	14 (13-17)	49 (45-55)	32 (31-35)	40 (38-43)	20 (19-22)	0.32 (0.30-0.35)	0.19 (0.18-0.21)	17 (16-19)	9 (8.5-10)
11	GANGARI (SI)	94 (92-98)	48 (46-51)	36 (33-39)	22 (21-24)	62 (60-64)	38 (36-40)	57 (55-60)	26 (24-29)	0.42 (0.40-0.44)	0.25 (0.23-0.27)	22 (21-25)	12.5 (11.5-13)
12	KASHIMPUR (G)	41 (38-45)	24 (23-25)	17 (16-18)	9.8 (9.2-10.6)	19 (18-20)	12 (11-13)	12 (11-13)	13 (12-15)	0.14 (0.13-0.15)	0.08 (0.07-0.09)	10 (9-12)	6.5 (6-7)
13	ATROULLI (G)	37 (34-41)	22 (21-24)	16 (15-17)	9.3 (9.0-9.8)	18 (16-21)	11 (10-13)	11 (10-12)	14 (13-16)	0.15 (0.14-0.17)	0.09 (0.08-0.10)	9.5 (9-11)	6.5 (6-7.5)
14	KALINADI-1 ST (SI)	105 (100-110)	52 (50-56)	39 (37-42)	25 (23-29)	65 (61-70)	37 (32-39)	60 (55-64)	27 (24-29)	0.46 (0.42-0.50)	0.26 (0.23-0.28)	26 (23-28)	13 (12-14)
15	KALINADI-2 ND (SI)	100 (96-110)	50 (49-52)	38 (36-40)	24.5 (24-26)	63 (60-67)	37 (34-39)	58 (55-62)	26 (24-28)	0.45 (0.42-0.48)	0.26 (0.22-0.30)	25 (24-28)	12.5 (12-14)
16	JALALI (PSI)	62 (58-64)	37 (35-41)	26 (24-29)	16 (15-18)	42 (41-44)	27 (25-28)	35 (33-38)	19 (18-21)	0.28 (0.27-0.30)	0.18 (0.17-0.21)	15 (14-17)	9.5 (9-11)
17	AKRABAD (PSI)	58 (54-62)	35 (33-37)	22 (21-24)	16 (15-17)	40 (38-44)	25 (24-27)	34 (62-37)	22 (21-25)	0.32 (0.30-0.35)	0.18 (0.16-0.20)	16 (14-17)	10 (9.5-11)
18	GABHANA (PSI)	62 (60-66)	39 (37-42)	26 (24-29)	17 (16-19)	42 (40-44)	28 (26-30)	36 (34-39)	21 (20-23)	0.33 (0.32-0.36)	0.20 (0.19-0.22)	18 (17-20)	11 (10-12)
19	KILA (SI)	116 (110-126)	59 (55-64)	40 (39-43)	26 (24-29)	69 (66-74)	49 (45-55)	60 (54-66)	27 (24-30)	0.48 (0.44-0.52)	0.30 (0.26-0.35)	24 (21-27)	13 (11-15)
20	CHANDAUS (SI)	96 (92-100)	55 (53-57)	37 (36-39)	23 (21-25)	59 (55-62)	40 (37-42)	52 (50-55)	24 (22-27)	0.40 (0.38-0.43)	0.19 (0.18-0.21)	16 (15-18)	9 (8.5-10)
21	PANATHI (G)	40 (38-43)	23 (22-25)	17 (16-19)	9.2 (8.8-9.5)	18 (17-20)	11 (10-13)	12 (11-13)	13 (12-15)	0.14 (0.13-0.16)	0.12 (0.11-0.13)	9.5 (9-11)	5.5 (5-6)

SI = Sewage irrigated soil; PSI = Partial sewage irrigated soil; G =Ground water irrigated soil; T= Total; E= DTPA- extractable

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RESULTS AND DISCUSSION

The results showed that there was no obvious change in soil pH values in sewage irrigation, partial sewage irrigation and ground water irrigation (Table-1). Soil organic matter was much higher in sewage irrigated soils than partial sewage irrigated soils or ground water irrigated soils. The CEC, an important index of soil holding cation nutrients capacity, significantly increased in sewage irrigated soils than in partial or ground water irrigated soils (Table 1). The total nitrogen content was $0.16 \pm 0.05\%$ in sewage irrigated soils and $0.13 \pm 0.03\%$ in partial sewage irrigated soils, which were much higher than $0.07 \pm 0.03\%$ in ground water irrigated soils. There was no distinct difference in the total P content in sewage irrigated soils and partial sewage irrigated soils but was much higher in sewage irrigated soils than ground water irrigated soils.

The average concentration of total heavy metals and DTPA extractable heavy metals viz. Cu, Mn, Zn, Cr, Cd, Pb and Ni are recorded in Table 2. The results showed that the concentrations of total heavy metals in sewage irrigated soils were about 2-2.4 times higher than that of ground water irrigated soils, while in partial sewage irrigated soils it was 1.6-1.8 times. The results also showed that concentration of total heavy metals increased with time in sewage irrigated and partial sewage irrigated soils. The lowest concentration of heavy metals in the top layer was in the month of September- October which may be due to leaching of metals

from top layer to lower depths. The concentration of DTPA extractable heavy metals showed no difference between partial sewage irrigated and sewage irrigated soils, which may be due to deposition of heavy metals in crops grown on the soils. There was a significant positive correlation between total heavy metal concentration, DTPA- extractable metal concentration, and soil-organic matter.

The data on bacterial, fungal and actinomycetes populations of the soils (Table 3) showed that number of bacterial and fungal colonies increased significantly with sewage water irrigation, maximum in sewage irrigated soils followed by partial sewage irrigated soils and least in ground water irrigated soils (Zhang *et al.*, 2008; Corstanje and Reddy, 2006). The fungal and bacterial population slightly increased with time. The increase in bacterial or fungal population may be due to mineralization of organic matter (Deshmukh *et al.*, 2010). The minimum population of actinomycetes was in sewage water irrigated soils (Tables 3). Soil organic matter was significantly positively correlated with soil bacterial and fungal population, and negatively correlated with actinomycetes. The results also showed (Table 3) that number of asymbiotic nitrogen fixing bacteria decreased with increase in bacterial population, denoting that mineralization of nitrogen decreased in presence of sewage. Sulfur-oxidizing bacteria ranges from 0.01×10^6 to 5.28×10^6 cells of per g of soil, only exception was Khairya contains 11.55×10^6 . Besides this some soils do not show specific growth and pH reduction was not in permissible level.

TABLE 3: Microbial population of collected soil samples (June, 2010 to April, 2011)

S.N.	Name of sample sites	Total bacteria count CFU $\times 10^7$ / of dry soil	Total fungal count CFU $\times 10^3$ /of dry soil	Total actinomycetes CFU $\times 10^3$ /of dry soil	Total nitrogen fixing bacteria CFU $\times 10^5$ /mL	Sulfur oxidizing bacteria MPN $\times 10^6$ / g soil
1	TAPPAL (PSI)	74 (58-78)	12 (10-14)	11 (9-15)	76 (65-85)	0.04 (ND-0.06)
2	KHAIR (SI)	168 (130-195)	22 (20-26)	9 (6-11)	80 (78-90)	0.94 (0.8-1.15)
3	LODHA (SI)	152 (140-187)	21 (19-23)	13 (12-15)	21 (9-33)	0.02 (ND-0.03)
4	MANDRAK (PSI)	68 (60-80)	13 (11-15)	14 (11-16)	13 (9-17)	0.02 (0.01-0.03)
5	IGLAS (PSI)	72 (66-84)	12 (10-14)	13 (11-15)	11 (4-20)	0.01 (ND-0.02)
6	SASANI (PSI)	84 (74-100)	14 (13-17)	32 (30-36)	14 (4-18)	ND
7	GONDA (SI)	150 (125-175)	24 (22-26)	20 (19-22)	35 (20-50)	0.25 (0.20-0.30)
8	KHAIRYA (SI)	178 (170-195)	25 (22-28)	23 (21-26)	33 (24-37)	2.12 (1.38-3.12)
9	MULLAPADA (SI)	172 (121-204)	27 (24-29)	38 (27-44)	27 (24-30)	4.21 (3.24-5.25)
10	DHANIPUR (PSI)	84 (54-110)	14 (12-16)	22 (20-24)	104 (90-111)	ND
11	GANGARI (SI)	154 (125-184)	23 (21-27)	34 (27-42)	23 (15-26)	0.04 (0.02-0.07)
12	KASHIMPUR (G)	100 (90-120)	10 (9-11)	26 (22-33)	48 (43-53)	ND
13	ATROULI (G)	120 (98-142)	12 (10-14)	12 (11-13)	20 (18-25)	ND
14	KALINADI-1 ST (SI)	248 (220-285)	29 (24-32)	16 (14-20)	16 (12-19)	0.46 (0.44-0.48)
15	KALINADI-2 ND (SI)	260 (235-295)	28 (24-30)	14 (13-19)	26 (14-29)	0.52 (0.46-0.56)
16	JALALI (PSI)	125 (110-140)	15 (12-18)	17 (15-18)	21 (18-24)	0.04 (0.03-0.07)
17	AKRABAD (PSI)	105 (95-125)	13 (11-15)	22 (21-24)	15 (12-19)	0.42 (0.34-0.52)
18	GABHANA (PSI)	122 (115-135)	14 (12-18)	14 (12-16)	8 (6-11)	ND
19	KILA (SI)	275 (245-300)	32 (30-35)	42 (33-56)	13 (10-15)	2.42 (2.12-2.77)
20	CHANDAUS (SI)	165 (140-185)	29 (28-32)	23 (14-33)	25 (21-28)	ND
21	PANATHI (G)	95 (85-108)	10 (9-12)	19 (12-24)	36 (30-40)	ND

SI = Sewage irrigated soil; PSI = Partial sewage irrigated soil; G = Ground water irrigated soil, ND = Non detectable

CONCLUSION

The application of sewage water for irrigation increases soil organic matter and CEC which in turn enhanced bacterial and fungal population. The application of sewage water also

increased total heavy metal concentration and nitrogen content in soils. The DTPA extractable metal concentration in SI and PSI soils were almost same suggesting that the heavy metals are deposited in crops grown on the soils,

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Biocontrol of ear rot fungi by plant growth promoting fluorescent Pseudomonads

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Abstract

In India, maize is the third most important food crop after rice and wheat but the productivity is severely affected as the plant is also prone to a number of diseases caused by fungi and bacteria. Ear rot caused by *Fusarium moniliforme* is one of the economically important soil and seed borne disease of maize and not easily controlled by chemical methods. Antagonistic bacteria may constitute an alternative for improving the crop productivity. In this study 13 fluorescent pseudomonads were isolated from rhizosphere of different plants and screened for plant growth promoting activities and biocontrol potential against *F. moniliforme* ITCC No. 2193. Among them a potential isolate, W3Gr-6(b) not only inhibited growth of *F. moniliforme* under in-vitro conditions but was also capable of maize growth promotion in presence of pathogen.

Key words: Maize, Ear rot, Pseudomonas, Bioformulation

1. Introduction

Maize (*Zea mays L.*) is one of the most versatile emerging crops having wider adaptability under varied agro-climatic conditions. It is cultivated on nearly 150 million hectare land in about 160 countries having wide diversity of soil, climate, biota and contributes 36% that is 782 metric tons (MT) in the global grain production (Plessis, 2003). In India, about 28% of maize produced is used for food purpose, about 11% as

livestock feed, 48% as poultry feed, 12% in wet milling industry (for example starch and oil production) and 1% as seed (Zaidi, 2010). Maize is often prone to several fungal pathogens. Among them *Fusarium* ear rot is the most damaging disease of corn (Davis, 1989; Nelson, 1992). *Fusarium* spp. cause diseases in corn such as ear rot (*F. moniliforme*, *F. verticillioides* and *F. proliferatum*), ear and stalk rot (*F. graminearum*), stalk rot (*F. verticillioides*), root rot (*F. graminearum* and *F. verticillioides*) and seedling blight (*F. graminearum* and *F. verticillioides*) (Munkvold, 2003). In India, *F. moniliforme* and *F. semitectum* are reported to be widespread in Uttar Pradesh, Punjab and Rajasthan (Lal and Dwivedi, 1982). For controlling the disease, farmers usually apply synthetic fungicides to the plants (Tagne, 2013). But reports reveal their post health and environmental issues which are very severe (Jacobsen and Backman, 1993). They can also increase the development of fungicide resistance to pathogens (Tarlochan, 2012). Now the application of microbes to control these phytopathogens is being explored as an alternative way to reduce the load of chemical fungicides and regarded as eco-friendly and cost-effective (Che and Jacob, 1994; Pal and Gardener, 2006; Khare and Arora, 2015). Among the bacteria applied in biocontrol of phyto-pathogenic fungi, fluorescent pseudomonads gained much attention of workers (Hass and Défago 2007; Mishra and Arora 2012; Tewari and Arora, 2014). They are major constituents of rhizospheric microbial flora and also show potential of plant growth

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promotion and biological control of different phytopathogens (Khare *et al.*, 2011; Bakhavatchalu *et al.*, 2012; Tewari and Arora 2014). Present study was performed to isolate some fluorescent pseudomonads having plant growth promoting and biocontrol potential against phytopathogen *F. moniliforme* and to evaluate their effect on maize growth enhancement and disease suppression.

2. Material and Methods

2.1 Bacterial isolates

Fluorescent pseudomonads were isolated from the rhizospheric soil of corn plants growing in farmers fields in vicinity of Kanpur (6.5°N, 80.3°E, 142 m above mean sea level), UP (India) on King's B (KB) medium. Colonies showing fluorescence under ultra violet exposure were further characterized by their cultural, morphological and biochemical characteristics (Garrity, 2005). Isolates were stored on KB slant for further use.

2.2 Phytopathogen fungus

Phytopathogen fungi *Fusarium moniliforme* ITCC No. 2193 was procured from IARI, Plant Pathology Division, New Delhi (India) and maintained on Potato Dextrose Agar (PDA) (HiMedia, Mumbai) for further use.

2.3 In-vitro screening of bacterial isolates for plant growth promoting (PGP) and biocontrol activities

Indole acetic acid (IAA) production was detected by the modified method as described by Brick *et al.*, (1991). Bacterial strains were grown without any shaking for 48 h on minimal medium amended with 0.2g/l L-tryptophan at 28°C. Fully grown cultures were centrifuged at

10000 rpm for 10 min at 4°C. The supernatant (1 ml) was mixed with two drops of orthophosphoric acid and 2ml of the Salkowski reagent. Development of pink color indicates IAA production. All isolates were screened on Pikovskaya's agar plates for phosphate solubilization. Determination of solubilization index (S.I.) was performed by measuring the halo (clear zone) diameter and the colony diameter, using the formula described by Edi-Premono *et al.*, (1996). Siderophore activity of the isolates was determined on Chrome-Azural S (CAS) medium. CAS plates were spot inoculated with bacterial strains and observed for development of orange halo against dark blue background around the colonies after 48 h of incubation at 28±2°C (Pérez-Miranda *et al.*, 2007).

All the isolates were also screened for the production of hydrogen cyanide by method described by Bakker and Schippers (1987). HCN induction media was prepared by adding 4.4 g glycine/l in King's B medium and bacteria were streaked on modified medium. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed inside the upper lid of petri plates and sealed with parafilm and incubated at 28±2°C for 4 days. After incubation color change (yellow to brown) in filter paper was visually assessed against control.

2.4 In vitro antifungal activity

A 10 mm disk of a pure culture of *F. moniliforme* was placed at the centre of a petri dish containing PDA. A loopful of bacterial isolate was streaked on PDA, 1.5 cm from the edge of each plate. Plate was cultured for 72h at 28°C and percent inhibition of radial growth (PIRG) was recorded by the following formula (Naureen *et al.*, 2010).



$$\text{PIRG} = (R1 - R2 / R1) \times 100$$

R1 = Radial growth of *F. moniliforme* in control plate

R2 = Radial growth of *F. moniliforme* interacting with antagonistic bacteria

2.5 Preparation of bacterial inoculum

For the preparation of bacterial inoculum, isolate W3Gr-6(b) with highest biocontrol activity was selected. This isolate was grown in 100 ml King's B broth on shaking incubator at 150 rpm for 48 h at $28^\circ\text{C} \pm 2^\circ\text{C}$ followed by addition of one percent carboxy methyl cellulose (CMC) as adhesive in aseptic conditions (Vidhyasekaran and Muthamilan, 1999).

2.6 Preparation of fungal inoculum

For this 8 mm disc from 14 days old culture of fungal pathogen, actively grown on a PDA plate, was transferred into potato dextrose broth aseptically and incubated on a shaking incubator for 5 days at $28 \pm 2^\circ\text{C}$.

2.7 Bacterial treatment in pot experiment

The W3Gr-6(b) isolate was tested for efficiency in promoting maize growth and controlling disease in pot experiment. For this maize seeds (Suvarna-589) were surface sterilized with 1 % sodium hypochlorite and washed 3 times with sterilized distilled water. Seeds were dried and coated with bacterial suspension and left whole night at $28 \pm 2^\circ\text{C}$ for proper mixing and coating with the bacterium. After 24 h, the bacterial suspension was drained off and the seeds were dried in shade for 30 min. For control untreated surface sterilized seeds were used (Vidhyasekaran and Muthamilan, 1999).

Pot experiments were conducted in BBAU campus, Lucknow (India) during the month of April-May. Earthen pot (30 x 24 x 16 cm) was filled with 5 kg of sterilized soil. Pots were mixed with 1% (w/w) of inoculum of *F. moniliforme*. Enough moisture was maintained by watering the pots regularly. Each pot was sown equidistance with 3 seeds. The treatments were: (1) Control (without any inoculation) (2) Negative control (with *F. moniliforme* inoculation) (3) W3Gr-6(b) (4) *F. moniliforme* + W3Gr-6(b). Each treatment was replicated three times.

After 14 days of sowing maize seedlings were observed for appearance of lesions and disease caused by *F. moniliforme* ITCC No. 2193. The number of dead or unemerged plants were also counted. On completion of 30 days plants were observed for growth enhancement by measuring root length, shoot length, fresh and dry weight.

3. Results

3.1 Bacterial isolates

On the basis of cultural, morphological and biochemical characteristics all the isolates were found to be fluorescent pseudomonads as described in Bergey's Manual of Systematic Bacteriology (Garrity, 2005) (Table 1).

3.2 In vitro screening of bacterial isolates for their plant growth promoting (PGP) and biocontrol activities

The production of IAA was highest by W3Gr-6(b) isolate followed by TS-42 and TS-43. Rest of the isolates showed very less amount of IAA production. All isolates were visually observed for determination of solubilization index (SI) by measuring the halo (clear zone) diameter and



the colony diameter. Amongst all W3Gr-6(b) showed maximum amount of phosphate solubilization and their P.S.I. was calculated to be 4.3. TS-42 and TS-43 also solubilized phosphate with P.S.I., 3.2 and 3.1, respectively. Production of siderophore was only detected in W3Gr-6(b) and TS-5. Amongst all isolates, TS-5 showed maximum HCN production, whereas, W3Gr-6(b) showed low cyanide production as it was evident by light yellow color. Rest of the isolates did not show cyanide production (table 2).

3.3 In-vitro antifungal activity

Amongst all isolates only six showed antifungal activity. W3Gr-6(b) showed maximum (57%) inhibition of radial growth of *F. moniliforme* ITCC No. 2193. Besides this GS-5, TS-41, TS-42, TS-43 and TS-44 showed 45, 38, 47, 42 and 41% growth inhibition respectively.

3.4 Bacterial treatment in pot experiment

Among the different treatments, maize seeds treated with W3Gr-6(b) bacterial suspension showed best results of plant growth parameters over untreated seeds (Table 3). W3Gr-6(b) isolate showed 118% increase in mean fresh weight, 179% dry weight, 32% root length and 42% shoot length compared to untreated control. Even in presence of pathogen, isolate W3Gr-6(b) showed 91% increase in mean fresh weight, 34% dry weight, 11% root length and 11% shoot length which was significantly higher than control. Treatment with pathogen only resulted in drastic decrease in all the parameters. The plants showed clear symptoms of ear rot.

Table 1: Biochemical characterization of the test isolates

Biochemical characters	GS-5	TS-4	TS-5	TS-6	TS-41	TS-42	TS-43	TS-44	W3Gr-4	W3Gr-6 (a)	W3Gr-6 (b)	W4HS
Fluorescent diffusible pigment	+	+	+	+	+	+	+	+	+	+	+	+
Gram reaction, cell shape	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Casease	+	+	-	+	-	-	-	-	+	-	-	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+



MR test	-	-	-	-	-	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis													
Starch	-	+	-	+	-	-	-	+	+	-	+	+	-
Lipid	-	+		+	-	+	+	+	-	-	+	+	-
Gelatin	-	+	-	+	-	+	+	-	+	+	+	+	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrate utilization													
Glucose	-	+	+	-	-	+	+	+	-	-	+	+	-
Lactose	-	+	-	+	-	-	+	-	+	+	+	+	+
Sucrose	+	+	-	+	-	-	+	-	+	-	+	+	-

Abbreviations: +, positive; -, negative

Table 2: Biocontrol activity of test isolates

S.N.	Name of Strain	HCN Production	Percent of radial growth inhibition (PIRG)*
1.	GS-4	-	Nd
2.	GS-5	-	45.00±0.034
3.	TS-4	-	Nd
4.	TS-5	+++	Nd
5.	TS-6	-	Nd
6.	TS-41	-	38.33±0.42
7.	TS-42	-	46.66±0.32
8.	TS-43	-	Nd
9.	TS-44	-	41.66±0.43
10.	W3Gr-4	-	41.35±0.39
11.	W3Gr-6(a)	-	Nd
12.	W3Gr-6(b)	++	56.89±0.28
13.	W4H5	-	Nd

*Values are mean of three replicates; ± SD
+++ (Excellent), ++ (Moderate), - Negative for traits, Nd Not detectable



Table 3: Effect of different treatments on growth parameters of maize after 30 days

Types of treatment	Dry weight (g)	Fresh weight (g)	Root length (cm)	Shoot length (cm)
Control	2.90±0.38 ^b	18.67±0.34 ^b	8.6±0.47 ^b	15.00±0.62 ^b
<i>F. moniliformae</i>	0.83±0.05 ^a	5.96±0.18 ^a	7.00±0.81 ^a	10.83±0.23 ^a
W3Gr-6(b)	8.10±0.14 ^d	40.87±0.15 ^d	11.33±0.95 ^d	21.33±1.2 ^d
<i>F. moniliformae</i> + W3Gr - 6(b)	5.53±0.68 ^c	25.03±1.2 ^c	9.60±1.20 ^c	17.83±0.62 ^c

Values are mean of three replicates. ±SD. Means in the columns followed by same letters indicates no significant difference ($P=0.05$) by Duncan's Multiple Range Test.

4. Discussion

Microbial inoculants play an important role in supplementing the essential plant nutrients for sustainable agriculture, economic and eco-friendly environment (Khare and Arora, 2014). (Maheshwari and Pandey, 2007; Arora *et al.*, 2014; Tewari and Arora, 2014). A wide variety of microbes have been utilized to enhance maize crop production (Gholami *et al.*, 2012) and efforts have been made to identify potential PGP and biocontrol bacteria. In the present study 13 isolates of fluorescent pseudomonads were screened *in vitro* for PGP and biocontrol activities against *F. moniliformae* causal organism of ear rot in maize. Ear rot results in great economic losses to farmers in India (Sharma *et al.*, 1993) and it is estimated to be causing damage of up to Use of fluorescent pseudomonads as bioinoculant has been reported (Weller, 2007; Gao, 2012). Studies confirmed their potential in P solubilization, siderophores production, secretion of lytic enzymes, and phytohormones, directly involved in plant growth promotion (Ardakani *et al.*, 2010). In this study isolate W3Gr-6(b) showed *in-vitro* production of IAA, solubilization of insoluble phosphate and siderophore production. These attributes

probably played a key role in maize growth promotion in *in vivo* conditions when seeds were coated with bacterial suspension. Our result showed that W3Gr-6(b) isolate increased growth parameters of maize to a significant level over untreated control. Earlier study by Chabot *et al.*, (1993) reported that *Pseudomonas* sp. caused a significant increase in maize plant height after 60 days. Isolate W3Gr-6(b) is a very promising IAA producer and contributed to 32% root and 42% shoot length increment in comparison to control. In a similar study Patten and Glick (2002) confirmed that the production of IAA by *Pseudomonas putida* GR12-2 plays a major role in the root development of canola (*Brassica rapa*) root system. Pan *et al.*, (1999) also reported that isolates of *Pseudomonas* (fluorescent) produced IAA that stimulates maize plant growth. In a study Khare and Arora (2009) reported dual effect of IAA in growth promotion and charcoal rot suppression of chickpea by *Pseudomonas aeruginosa*. Another PGP activity of W3Gr-6(b) isolate was P solubilization, that influences plant growth by providing soluble form of phosphate (Khan and Joergesen, 2009). W3Gr-6(b) also showed siderophore production. A number of workers have reported the production and role of



siderophore produced by pseudomonads in controlling phytopathogens (Bouizgarne, 2013).

Crop loss due to ear rot caused by *F. moniliforme* is globally reported (Scaufaire *et al.*, 2011; Wang *et al.*, 2014) and current practice used in eradication involves chemical pesticides. Fluorescent pseudomonads produce metabolites such as antibiotics, siderophores and hydrogen cyanide (HCN) as mechanism of biocontrol (Weller, 2007) and commercially adopted in controlling several plant pathogens. Studies indicate that fluorescent and non-fluorescent *Pseudomonas sp.* directly or indirectly control soil borne pathogen including *F. moniliforme* (Hebbar *et al.*, 1992). In a study Pal *et al.*, (2001) reported suppression of maize root diseases caused by *Macrophomina phaseolina* and *F. graminearum* by PGP isolate of a fluorescent *Pseudomonas sp.* EM85. Recently Yasmin *et al.*, (2014) also confirmed that the *P. aeruginosa* Z5 has biocontrol activity against *F. oxysporum*, *Fusarium sp.* In our investigation W3Gr-6(b) also inhibited the growth of *F. moniliforme* ITCC No. 2193 both *in vitro* and *in vivo* conditions.

In this way it can be recommended that use of fluorescence pseudomonads in the form of bioinoculants may be done in stimulating yield and growth of maize crop in field conditions. Besides this, further studies on its field performance in eradication of fusarium ear rot will be done before developing the biopesticides targeting crop and phytopathogen in field conditions.

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Viewpoint

Prospecting the roles of metabolites and additives in future bioformulations for sustainable agriculture

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ABSTRACT

Addition of beneficial microorganisms as plant growth promoting (PGP) rhizobacteria (PGPR) and fungi (PGPF) for augmenting plant growth and inhibiting phytopathogens is now more than a century old story, but still we are only in the lag phase concerning the use of microbe based products in agriculture. The benefits of microbe based products, known by various names such as biofertilizers, biostimulants, bioinoculants, biopesticides, bioformulations, certainly are huge in respect to the sustainability they provide and being eco-friendly. Still the constraints associated with quality, reliability and performance are hampering their progress in the market. Due to these constraints the bioformulations are lacking far behind the chemicals. Hence, new avenues and directions have to be explored so as to remove the associated problems and instigate belief amongst the end users/farmers. Current research is already showing the trends for the development of formulations which will be more reliable and consistent. Inoculation of PGP microbes along with metabolites is showing great promise, proving to be more effective with multiple roles and hence showing the way for the development of future bioformulations.

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1. Introduction

Soil is the richest habitat for microorganisms on earth. Most of these microorganisms, particularly those inhabiting the rhizosphere are beneficial to the plant and ecosystem as a whole. However, anthropogenic activities and indiscriminate use of chemicals in the form of fertilizers and pesticides caused major impact on the soil fertility, diversity, and even on health of animals and humans. Moreover, production of fertilizers and pesticides itself is resulting in issues such as greenhouse gas emission and environmental pollution. The need is to go for the biological alternatives to enhance crop productivity and replace the harmful chemicals as soon as possible. Human population is growing swiftly and is expected to cross nine billion by 2050 (FAO, 2012). However, the arable land is not going to increase. In fact, the use of chemicals has only resulted in arid and non-fertile soils. It is well known now that the uninhibited use of chemicals has resulted in loss of beneficial soil microorganisms (Seneviratne and Kulasoorya, 2013). The eco-friendly bioformulations, however, have not taken over the agro-market because of constraints associated with

them. The limitations include the maintenance of microbial populations and vigour, erratic performance in the field, low organic carbon in the soil, inconsistent quality and poor shelf life (Arora, 2015). There are some questions to be answered so as to gain the confidence of the end users and make our agriculture system free of toxic chemicals. To find the solutions it is necessary to learn from the work done in the field and also know the direction in which the current research is moving so as to predict the future course to develop better bioinoculants. The amendment of metabolites along with PGP microbes can be far more effective in comparison to traditional cell based formulations.

2. Role of metabolites in future bioformulations

Bioformulations are defined as products containing microbes or their metabolites which can be used for providing nutrients or bioregulators or combat phytopathogens (Arora et al., 2010). Bioagents, used for bioformulation production, are smart microbes generally found in the rhizosphere, affecting the plant growth directly or indirectly by diverse mechanisms (Lugtenberg and Kamilova, 2009). Microbes belonging to genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Pseudomonas*, *Bacillus*, *Azotobacter*, *Azospirillum*, *Trichoderma*, and mycorrhizal fungi are generally used in bioformulation production. Apart from *Bacillus thuringiensis* (Bt),

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Role of Beneficial Soil Microbes in Sustainable Agriculture and Environmental Management

Jitendra Mishra¹ • Jai Prakash² • Naveen Kumar Arora^{3*}

Abstract Rapidly increasing human population is expected to make food security a big issue in the future. Agriculture is facing severe challenges of land degradation, lesser productivity and susceptibility towards a biotic and biotic stresses. Sustainability in the agricultural sector is proving a formidable task because the current trend involves excessive use of chemical fertilisers and pesticides. Besides these, anthropogenic activities such as urbanisation and industrialisation are generating more waste that is posing a severe threat to the ecosystems. Various approaches were being tried to eradicate waste and restore ecosystems, but the success was limited. Beneficial soil microbes (BSMs) are identified as suitable candidates that may help in the sustainable management of the environment. These microorganisms possess several mechanisms that can be exploited at the commercial level in developing biotechnology for solving the key environmental issues. Beneficial microbe-based products currently used in agroecosystems have shown a remarkable success. Their proper use in agroecosystems is changing the scenario of present-day agriculture. In the future, utilisation of such microbes in the clean-up of pollutants, waste eradication and combating climate change can provide substantial aid in on-going greener campaign towards environmental sustainability. In this review, we have summarised the role of BSMs particularly the plant-growth-promoting bacteria and fungi in sustainable agriculture and also addressed their role in the management of environmental problems.

Keywords Beneficial soil microbes, Pollutants, Climate change, Agroecosystems, Sustainability, Agriculture, Plant Growth Promoting Rhizobacteria

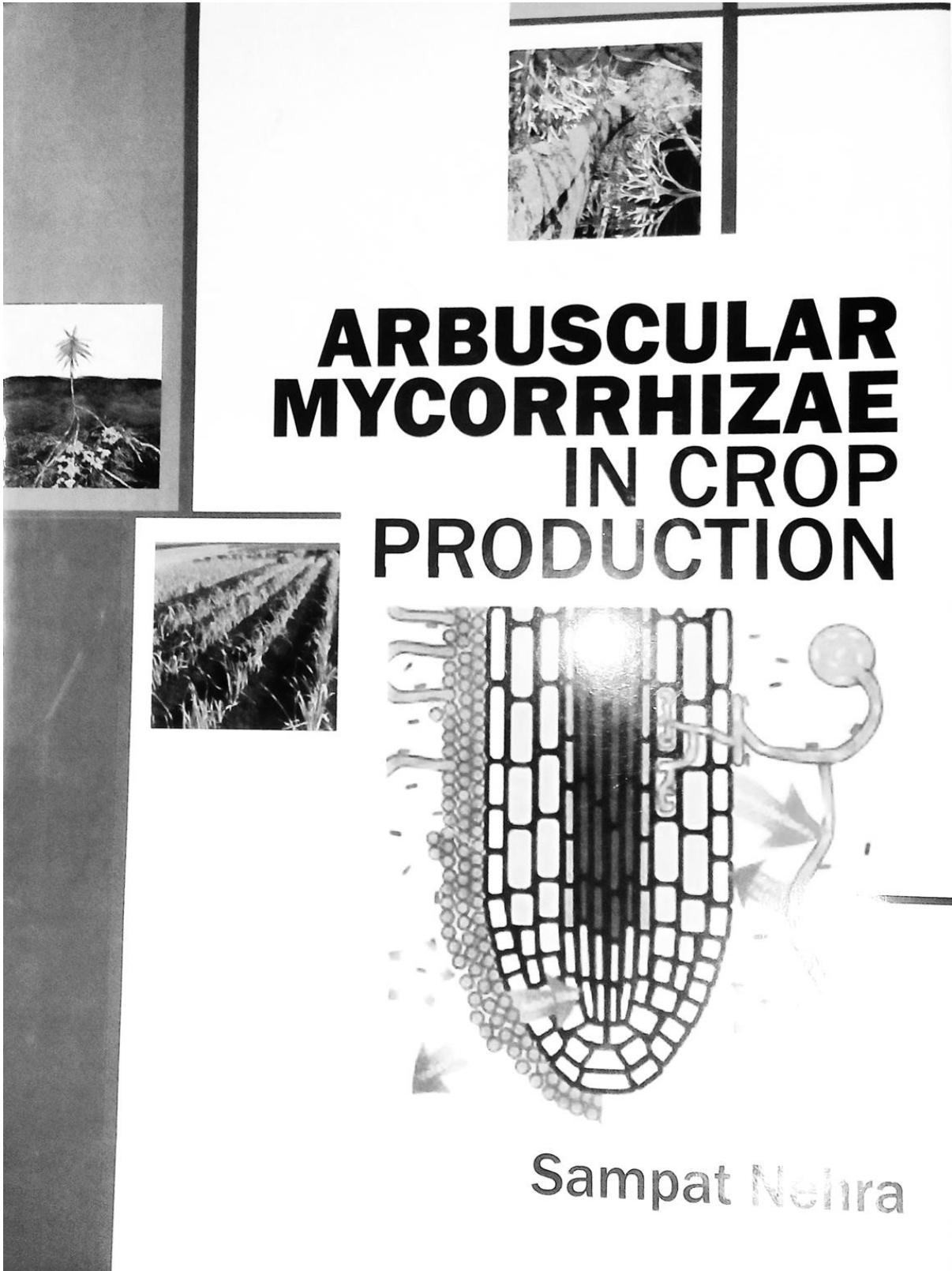
1. Introduction

The human population is increasing very rapidly and creating excessive pressure on the existing land area for food,

fuel and raw materials. To supply the demand, agriculture practices are using intensive amounts of chemical fertilisers and pesticides which has resulted in land degradation and loss of biodiversity (Carsten and Mathis, 2014). In many developing countries, agriculture plays a vital role in the economic development and also provides self-employment (Gindling and Newhouse, 2012). According to an estimate, it was found that developing countries such as India, Pakistan, Bangladesh, Iran, Afghanistan, Nepal, Sri Lanka and Bhutan are losing crops, equivalent to at least, US\$10 billion annually due to land degradation (Lal *et al.*, 2010). Several factors have been reported by the researchers, which are responsible for degradation of soils. Among them, anthropogenic activities are directly involved in the land degradation process. The result of land degradation, may be exhaustion, salinisation and desertification of soil (Kesavan and Swaminathan, 2008). Many technological inventions are being carried out for the improvement of crop productivity, but still, we are not in a position to fulfil the global demand for food. Microbes associated with plants can be used to overcome the problems related to soil salinity, fertility, degradation and habitat loss. Soil contains diverse organisms like bacteria, archaea, fungi, algae, insects, annelids and other invertebrates which show an intimate relation to each other and with plants (Glick, 2010). Among them, microbial entities are unique as they are directly involved in increasing soil fertility, plant growth promotion and lowering biotic and abiotic stresses by various mechanisms which are known as BSMs. BSMs enhance the plant growth by nutrient uptake, form complex soil matrix and help in plant defence response by secretion of various metabolites. In addition, BSMs can show tolerance towards adverse environmental conditions such as salt stress, drought stress, weed infestation, nutrient deficiency and heavy metal contamination. Since past few years, researchers have found that soil microbes play beneficial and harmful roles in the soil ecosystem. However,

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ARBUSCULAR MYCORRHIZAE IN CROP PRODUCTION

Sampat Nehra

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ROLE OF MYCORRHIZAE IN SUSTAINABLE AGRICULTURE

JITENDRA MISHRA AND RACHNA SINGH

Sustainable agriculture is the prime need of our society. Microbial technology based approaches has been proved successful and somehow helping to achieve to goal of sustainability. Fungal communities in agro- ecosystem has both destructive as well as supportive role whereas obligate association of fungi with host plant in form of mycorrhiza is key solution of various soil related problems. This evolutionary relationship is know from millions of years and its role in agriculture has been appreciated as the association has countless capabilities to promote plant growth and defense from various ailments and regarded better alternative of chemical fertilizer and pesticides.

After too many technological advances in the field of agronomy, the required demand cannot be accomplish whereas, conventional agriculture is no longer suited to feeding humans and preserving ecosystems (Eric *et al.*, 2008). The agricultural practices solely depend on the chemical fertilizer and pesticide to enhance the productivity. Although by using such synthetic chemicals we somehow get succeed in increasing crop productivity but this also poses a devastating effect on agricultural land and results in loss of soil fertility, lesser crop yield, development of resistance in pest. That is why there is need to focus on such agricultural practices which are more eco-friendly and sustainable. So the ultimate requirement is developing sustainable agricultural practices. Sustainable agriculture may be defined by various ways: it is the adaptive capacity of agriculture to adapt to future changes (Gafsi *et al.*, 2006) or to maintain long term crop productivity (Ikerd, 1993). whereas somewhere it addressed a set of management strategies in societal concerns about food quality or environment protection (Franciset *et al.*, 1987, Boiffin *et al.*, 2004). Role of microbes in agriculture have been known from a century and both positive and negative aspects are of much concern. In last few years various microbes mediated phenomenon occupied a central position among the

Naveen Kumar Arora *Editor*

Plant Microbes Symbiosis: Applied Facets

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Biopesticides: Where We Stand?

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Jitendra Mishra, Sakshi Tewari, Sachin Singh,
and Naveen Kumar Arora

Abstract

Chemical pesticides are well known for their effective role in disease management because not only they act on a broad host range but production technology is also less expensive. However, the devastating part is their huge negative impact on the environment including the living beings of the planet. In spite of this, in the absence of suitable alternative, the use of synthetic pesticides has dominated around the globe. By the advent of greener approach of developing and using biopesticides, the situation is gradually changing but in fact can move far more swiftly in this direction which will be sustainable and eco-friendly. Although biopesticides are slowly replacing the chemical pesticides, a complete global look at the scenario indicates that the former and particularly the industries based on them are still in an insecure position in comparison to the chemicals which rule the agriculture. We can say that the biopesticides, although show a great promise, have not come up to the desired level so as to displace the dominance of chemicals. In this chapter, the global scenario of biopesticides is discussed emphasizing upon the current demand, use, constraints, and remedies.

Introduction

Two-thirds of today's world population depends upon agriculture for livelihood, but nowadays, growth and production of agricultural crops are

getting hampered day by day (Elumalai and Rengasamy 2012). When farmers see their agricultural crops declining in yield and production, they often expect a dramatic, magical treatment to make them lush, green, and healthy again, so that the productivity increases. As a result, they start using chemical pesticides, disregarding their future effects. The extensive use of these synthetic organic chemicals in the past decades has led to a number of long-term environmental problems (Arora et al. 2012). Keeping all these

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Raffaella Balestrini *Editors*

Bioformulations: for Sustainable Agriculture

 Springer

Bioformulations for Plant Growth Promotion and Combating Phytopathogens: A Sustainable Approach

1

Jitendra Mishra and Naveen Kumar Arora

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Abstract

The role of microbes in sustainable agriculture has provided new insights to agro-economy, and one of the direct benefits is the lesser reliance on chemical fertilizers and pesticides as the continuous application of these chemicals not only showed detrimental effects on agro-ecosystems but also resulted in health risks to humans and animals. In last few years, the development of microbial bioinoculants for enhancing plant growth and disease eradication has emerged as an alternative, but a broader aspect of their application as formulatory product has remained in infancy especially in developing countries. At the economic and social level also, this green strategy is facing hurdles and lags far behind their competitors, the synthetic fertilizers and pesticides. Most of the times it has been found that bioformulations available for a particular crop do not give good results equivalent to those in the laboratory conditions. Such and related constraints are major challenges of this greener approach. Various workers all over the world are continuously engaged in developing formulation products which could be easier to use, show enhanced activity toward phytopathogens, and may cover more target crops. Whole process of bioformulation development, from screening of microbe to product development and its implementation, need to be reviewed.

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Regulation of Biopesticides: Global Concerns and Policies

16

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

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Abstract

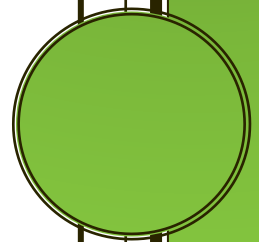
In the last few years, biopesticides have emerged as suitable alternatives to synthetic chemical pesticides. They are cheaper and pose no threat to agroecosystems. This is why their demand and production are also increasing at global level. The law and policies regulating their use and development vary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

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APPENDIX



11. APPENDIX

(Media Used)

King's B Agar

Composition	Amount
Protease Peptone	20.0g
Magnesium Sulphate Heptahydrate	6.0g
Glycerine	15.0 ml
K ₂ HPO ₄	2.50 g
Distilled water	1000 ml
Agar	20.0g
pH	7.2±0.2

Nutrient Agar

Composition	Amount
Peptone	5.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2±0.2

Motility Agar

Composition	Amount
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	3.5 g
Distilled water	1000 ml
pH	7.2±0.2

Simmons Citrate agar

Composition	Amount
Ammonium dihydrogen phosphate	1.0 g
Dihydrogen phosphate	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
Magnesium sulphate	0.2 g
Bromothylol blue	0.8 g
Agar	15.0 g
Distilled Water	1000 ml
pH	7.2±0.2

Tryptophan broth

Composition	Amount
Casein enzymic hydrolysate	10.0 g
Sodium chloride	5.0 g
DL-Tryptophan	1.0 g
Distilled Water	1000 ml
pH	7.5±0.2

MR-VP Broth

Composition	Amount
Peptone	7.0 g
Dextrose	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1000 ml
pH	6.9±0.2

OF medium

Composition	Amount
Casein enzymic hydrolysate	0.20 g
Phenol red	0.003 g
Agar	0.300 g
Distilled water	1000 ml
pH	7.4±0.2

Nutrient gelatin

Composition	Amount
Peptic digest of animal tissue	5.0 g
Beef extract	3.0 g
Gelatin	120.0 g
Distilled water	1000 ml
pH	6.8±0.2

Starch Agar

Composition	Amount
Peptone	10.0 g
Sodium chloride	5.0 g
Calcium chloride dihydrogen	0.1 g
Tween 80	10.0ml
Agar	20.0 g
Distilled water	1000 ml
pH	6.0±0.2

Skim milk agar

Composition	Amount
Skim milk powder	100.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled Water	1000 ml
pH	7.2±0.2

Lipase agar medium

Composition	Amount
Peptone	10.0 g
Sodium chloride	5.0 g
Calcium chloride dihydrogen	0.1 g
Tween 80	10 ml
Agar	20 g
Distilled water	1000 ml
pH	6.0

Trypticase soy agar

Composition	Amount
Pancreatic digest of casein	15g
Papaic digest of soyabean meal	5.0 g
Calcium chloride	5.0 g
Agar	20 g
Distilled water	1000 ml
pH	7.3±0.2

Christensen's Urea Medium

Composition	Amount
Peptic digest of animal tissue	1.0 g
Dextrose	1.0 g
Sodium chloride	5.0 g
Disodium phosphate	1.2 g
Monopotassium phosphate	0.8 g
Phenol red	0.012 g
Agar	15 g
Distilled water	1000 ml
pH (at 25°C)	6.8±0.2

Pikovskaya's agar medium

Composition	Amount
Yeast extract	0.5 g
Dextrose	10 g
Calcium phosphate	5.0 g
Ammonium sulphate	0.5 g
Potassium chloride	0.2 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.0001 g
Ferrous sulphate	0.0001 g
Agar	15 g

Potato Dextrose Agar

Composition	Amount
Potatoes, infusion from	200 g
Dextrose	20 g
Agar	15 g
Final pH (at 25°C)	5.6±0.2

Succinate Broth

Composition	Amount
K ₂ HPO ₄	6.0 g
KH ₂ PO ₄	3.0 g
(NH ₄) ₂ SO ₄	1.0 g
MgSO ₄ .7H ₂ O	0.2g
Succinic acid	4.0 g
pH	7.0±0.2