"Selection and utilization of Fluorescent Pseudomonads for enhancing production of sunflower crop in arid soil infested with Macrophomina phaseolina"

THESIS

SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW
प्रज्ञा शील करुणा
ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted by

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Enrolment No: 383/11

UNDER SUPERVISION OF

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BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY, NAAC ACCREDITATION 'A' GRADE)
LUCKNOW-226 025

2015

Dedicated to

My Beloved Parents

and

Supervisor

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Certificate

This is to certify that the work embodied in the thesis entitled "**Selection and utilization of Fluorescent Pseudomonads for enhancing production of sunflower crop in arid soil infested with *Macrophomina phaseolina***" has been carried by **Ms. Sakshi Tewari** for the award of degree of Doctor of Philosophy in Environmental Microbiology under my supervision. She has fulfilled the requirements of academic ordinance of Babasaheb Bhimrao Ambedkar University, Lucknow, for the award of degree of Doctor of Philosophy in Environmental Microbiology. It is further certified that all the data given in this thesis are her own observations and are genuine. To the best of my knowledge her thesis work is original and has not been submitted anywhere for the award of other degree.

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Declaration

This is to certify that I have worked on the research thesis entitled **"Selection and utilization of Fluorescent Pseudomonads for enhancing production of sunflower crop in arid soil infested with *Macrophomina phaseolina*".** 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.

Place

Date **Sakshi Tewari**

Acknowledgements

The outmost praises goes to Lord Almighty for bringing me into this world and showering onto me all the blessings. You provided me with opportunity when I was pathless, and empowered me with qualities of patience, strength and confidence which helped me to undertake my research work with more purpose. Without your blessings, I would not have been writing this last note in my research work. Thank you for everything.

During the period of my research, I have been blessed with some wonderful people who have spun a web of support around me. Words shall never be enough to express how grateful I am to these incredible people in my life that made this thesis a reality. I would like to make an attempt to thank them one by one, reminiscing the time I have spent with them and which for sure I will treasure all my life.

I am deeply indebted to my mentor, **Dr. Naveen Kumar Arora**, Coordinator, Department of Environmental Microbiology, BBA University, Lucknow for the affectionate, sustained and encouraging guidance. He was always accessible to me and took off time from his busy schedule, every time I needed his guidance in my research work. Each discussion with him provided me with invaluable insights and broadened my thought process. There were times when I was short of ideas, thoughts and impatience took over me. In those difficult times, he guided me with his invaluable suggestions, lightened up my way and encouraged me to move ahead. From him, I have learned to keep calm in difficult times, think critically, select problems and to solve them in a manner which is ethical and acceptable. This has not only made me to perform my duties as a responsible researcher but has also made me a good human being. I truly owe him what I am today. I would like to thank him for furthering my knowledge in diverse areas of Microbiology. He has always encouraged me to participate in conferences/seminars and it is because of his foresight, that, today I have a handful of awards to my name. It was because of his initiatives, that, I was able to attend various National and International conferences and could deliver presentations of my research work. This gave me a chance to expose myself to national and international competence and thus hone my skills as a researcher. His drive for scientific excellence has always pushed me to aspire for more in research and has taught me to show humility, so that my appetite for good research always remain incomplete. It was a great pleasure for me to have a chance of working with such a knowledgeable and intellectual guide. He was the best choice I could have made for a supervisor.

I salute the efforts of honourable Vice Chancellor, **Professor R C Sobti** for his invaluable support to research and researchers, in the University. Without his support and leadership I would not have been able to bring such a graceful completion of my research work. I would like to thank him for all the support provided to me while I was a student in the department.

I would also like to express my sincere gratitude to **Prof H K Sehgal**, former Vice Chancellor, CSJM University, Kanpur, for providing laboratory facilities at CSJM University. I owe my profound gratefulness to **Prof D P Singh**, Dean, School for Environmental Sciences, for his support and providing facilities in University Science Instrumentation Centre, BBA University, Lucknow.

I would like to address special thanks to **Dr Rajesh Kumar, Dr V S Baghel, Dr Jay Shankar Singh and Dr R N Bhargawa**, faculty members of the Department of Environmental Microbiology for support and cooperation. I also thank Prof H B Singh, Head, Department of Mycology and Plant Pathology, BHU, Varanasi for being particularly supportive during times of need. I also thank Dr Ram Chandra Scientist F, IITR, Lucknow for his valuable support.

I will be unfair on my part, if I don't acknowledge some of my friends with whom I have shared my research experiences since it were a joy and enlightenment to me. I was fortunate to have friends and collegues like Dr Ekta, Mr Sachin, Mr Govind, Mr Jitendra, Ms Rachna, Ms Sweta, Ms Maya and Mr Jai Prakash who opened their heart out every time I was stuck into problems. When I felt dejected they always stood beside me and gave me strength and confidence to fight all odds. I would only like to say u all that when I will step out of this University not only I will be carrying a degree but also wonderful memories I have shared with you all, which I will treasure all my life. I am also thankful to the staff members Mr Saheb Sharan, Mr Sartaj, Ms Nancy and Mr Dheeraj for providing help and support whenever needed.

I am also thankful to **Mrs Preeti Arora, Pranay Arora** and **Nav Arora** for affectionate support during the course of my research work. I would express my deep sense of gratitude to the patience, affection and support shown by my grandmother Smt Radha Tiwari. I take this opportunity to dedicate this work to my parents **Mrs Vandana Tiwar**i and **Mr AK Tiwari** who have made me what I am. Credit is due to my in laws **Mrs S L Mishra**

and Mr P N Mishra for their moral support, blessings and everlasting inspiration that always is a mobilizing force in the completion of this work. I would also like to thank my brother **Mr Siddharth Tiwari** who stood by me in all thick and thins, and have given consistent support throughout my research.

Last but not the least the warmest thanks to my friend cum husband **Mr Anurag Mishra** for his understanding and patience while I was far away from home during the period of my research. He has supported me in each and every way and inspired me in all dimensions of life.

All of you just made it possible for me to reach this last stage of my endeavor. Thank You from the deepest corners of my heart.

Place **:** **(Sakshi Tewari)**

Date :

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Notations and Abbreviations

°	degrees
′	minutes
%	percent
°C	degree centigrade
λ max	maximum wavelength
Ar	absorbance of reference
As	absorbance of sample
R_T	retention Time
N	north
E	east
W	weight
h	hours
m	metre
cm	centimetre
mm	milimetre
um	micrometre
nm	nanometre
g	grams
mg	milligrams
ug	micrograms
ng	nanograms

L	liter
mL	mililiter
uL	microliter
M	molar
mM	millimolar
N	normal
kb	kilo base
V	voltage
w/v	weight by volume
v/v	volume by volume
min	minutes
Ha	hectare
PGP	plant growth promotion
PGPR	plant growth promoting rhizobacteria
YIB	yield increasing bacteria
PGPB	plant growth promoting bacteria
PHPR	plant heath promoting rhizobacter
CSSRI	central soil salinity research institute
GOI	government of india
DNA	deoxy ribonucleic acid
rRNA	ribosomal ribonucleic acid
EPS	exopolysaccharide
CFCS	cell free culture supernatant

DW	distilled water
UV	ultra violet
OD	optical density
HCN	hydogen cyanide
$MgSO_4$	magnesium sulphate
K_2HPO_4	dipotassium phosphate
$CoCl_2$	cobalt chloride
$ZnSO_4$	zinc sulphate
$NiCl_2$	nickel chloride
$MnSO_4$	manganese sulphate
NaOH	sodium hydroxide
$ZnCO_3$	zinc carbonate
H_2SO_4	sulphuric acid
$FeSO_4$	ferrous sulphate
KBr	potassium bromide
KNO3	potassium nitrate
NaCl	sodium chloride
H_2O	water
$(NH_4)_2SO_4$	ammonium sulphate
H_3BO_3	boric acid
$CuCl_2$	copper chloride
$NaMoO_4$	sodium molybdate
HCl	hydrochloric acid

ZnO	zinc oxide
$MgCl_2$	magnesium chloride
$CaCO_3$	calcium carbonate
EDTA	ethylenediaminetetraacetic acid
NaClO	sodium hypochlorite
USA	United States of America
EU	European Union
spp	species
SA	salicylic acid
CFMB	cell free metabolite bioformulation
KB	king's B
DMM	davis minimal media
SI	solubilization index
CAS	chrome-azurol s
TLC	thin layer chromatography
PDA	potato dextrose agar
RT	room temperature
CMC	carboxy methyl cellulose
rpm	rotations per minute
SEM	scanning electron microscopy
IMC	inherent moisture content
CFU	colony forming unit
DAS	days after sowing
TCP	tricalcium phosphate

IAA	indole-3- acetic acid
CMM	chitin minimal medium
RDP	ribosomal database project
SDS	sodium dodecyle sulphate
FTIR	fourier transform infrared spectroscopy
HPLC	high performance liquid chromatography
WHC	water holding capacity
dS	decisiemens
DMRT	duncans multiplicity test range

Chapter 1

Introduction

Agriculture is considered to be the most vulnerable sector, mainly, due to its dependence on various biotic and abiotic factors. Often these factors have not only affected the crop yields but have also thrown millions into poverty and malnourishment. Asian and African people have been and are particularly affected due to this vulnerability. Though productivity in agriculture, at local levels, generally gets affected due to erratic rainfall, seasonal variation and other abiotic stress as, but, more recently, salinization has erupted as a global phenomenon, that has indiscriminately affected agriculture world over. Soil salinity in the recent year is becoming major concern for agriculturists and governments across the globe as it results in stagnation of productivity of major crops (Arora et al. 2012; Nadeem et al. 2015). In arid and semiarid regions, soil salinity is considered the most significant cause of land degradation. It is an active process, spreading globally in more than 100 countries and covering on an average more than one billion hectares (ha) (Kumar et al. 2015).

At present more than 900 million ha land worldwide is suffering from the problem of salinity stress (Flowers 2004; Khan and Panda 2008; Tewari and Arora 2014a). Salinity is the principal cause of crop failure worldwide, dipping yield of major crops by more than 50 % (Mahajan and Tuteja 2005). It should be highlighted that with ever increasing population demand for food is expected to rise by 3-5 times and for this the current food production has to increase by 60 % in order to meet the requirements of the future (Wild 2003). According to the Food and Agricultural Organization (FAO 2008), if corrective measures are not taken then salinization of arable land may result in 30 % land loss in the next 25 years and up to 50 % loss by the year 2050, which may cause loss of hundreds of millions of dollars each year due to reduction in crop productivity and crop failure (Munns 2005; Arora 2015).

The probability of occurrence of extreme climatic events has increased the incidence of salinization stress in last couple of decades, and farmers lack the management options to sustain the agricultural productivity (Kalra et al. 2013). When farmers see their agricultural crops declining in yield and production due to stresses they often expect a dramatic and magical treatment to make them lush, green and healthy so that productivity increases. As a result, they start using chemicals and fertilizers disregarding their future effects. The extensive use of certain synthetic organic chemicals in the past decades has led to a number of long-term environmental problems (Mishra et al. 2015). Conventional breeding has been able to meet the problem so far, but time has come to search for alternative means to increase the pace of crop productivity to meet the projected demand for agricultural products (Cooper et al. 2009). Improvement in the genetic base of the crop plants for better adaptability to the abiotic and biotic stresses is a time-consuming process and improving all crop plants to face the challenge of abiotic stress equally at the same time is very unlikely (Sharma et al. 2013). In order to meet the increasing demand of food supply, better adaptation of the crop plants to abiotic stresses as well as geographical expansion of agricultural lands by making unusable lands usable for crop production are the key necessities. In this regard, useful soil microbes may become very handy and provide a quick-fix solution to the problem (Tank and Saraf 2010; Devi and Momota 2015).

One of the recent focuses of research involves application of plant growth promoting rhizobacteria (PGPR) to combat these stresses. PGPR are a group of bacteria that can actively colonize plant roots and increase plant growth. They also possess many attributes which may help in solving various environmental problems (Kloepper and Schroth 1978). PGPR are beneficial soil bacteria, which may facilitate plant growth and development directly or indirectly (Glick 1995). Direct stimulation may include benefits to the plant such as fixed nitrogen, phytohormones, sequestered iron by bacterial siderophores, and soluble phosphate, while indirect plant stimulation is attributed to biocontrol (antagonistic interrelations with soil-borne phytopathogens) (Glick and Bashan 1997). The different mechanisms by which PGPR inhibit pathogens include production of lytic enzymes, HCN, volatile organic compounds, antibiotics, etc (Hayat et al. 2010).

The intimate relationship of PGPR with plants has been a long-established theory, but the focus of this study is to apply these bacteria in agronomy to mitigate salinity stress, for which the information is meager. Till date, many bacterial genera, such as *Alcaligenes, Azospirillum, Azotobacter, Bacillus, Clostridium, Klebsiella, Pseudomonas, Rhizobium, Thiobacillus, Serratia*, *Streptomyces* etc which work as plant growth promoters are being used and tested but their role role under salinized conditions in presence of pathogen is still needed to be explored (Whipps 2001; Arora et al. 2013). Various researchers aim to develop salt-tolerant crops to overcome the effect of salinization but this approach is uneconomical and time consuming whereas, use of growth favourable microbes to alleviate salt stress is a better solution (Arora et al. 2012). The use of PGPR to alleviate salinization is becoming one of the most promising approaches to enhance production and yield of diverse crops in salinity-affected regions.

Amongst the most extensively studied PGPRs are the strains of *Pseudomonas.* Members of genus *Pseudomonas* owe a lot of attention because of their intrinsic ability to colonize the rhizosphere at high density, to compete successfully with microorganisms, and to produce secondary metabolites with powerful antimicrobial activity. Among them fluorescent Pseudomonads have received particular attention throughout the globe because of their catabolic versatility, excellent root colonizing ability and capacity to produce wide range of metabolites that favor the plant withstand under varied biotic and abiotic stress conditions (Mayak et al. 2004; Selvakumar et al. 2015).

Fluorescent pseudomonads are one of important rhizospheric bacteria which are being utilized as biocontrol agents with definite growth-promoting activities (Pandya and Saraf 2015). A number of highly effective disease-suppressive agents are produced by them, making this group of bacteria the most widely studied group of the rhizosphere (Nagarajkumar et al. 2004; Meera and Balabaskar 2012). However, little is known about the mechanism by which pseudomonads promote growth of plants and suppress diseases under saline stress conditions. The mechanisms by which these organisms offer beneficial attributes such as antagonism against fungal pathogens of agricultural crops and nutrient recycling in the rhizosphere microcosm under salinity condition need to be researched. Large amount of work has been done on pseudomonads taking diverse set of crops, but its mechanism of action under saline conditions along with sunflower (*Hellianthus annuus*) and its dreadful phytopathogen, *Macrophomina phaseolina* has not been studied much.

Oilseeds, including sunflower have assumed significant importance as agricultural crops in India since time immemorial. India has the second largest area under oilseeds cultivation, next only to USA. However, it falls behind (from earlier sixth) to ninth place in terms of production due to comparatively low yields (Commodity profile 2015). Sunflower is a major edible oilseed crop after groundnut at the global level (Swamy et al. 2010). It is considered as the best diversified kharif (oil seed) crop of the Uttar Pradesh (Chand and Jha 2001).

In view of the economic importance of oilseed crops, particularly sunflower, in India and also in other countries, the need is to increase the yields, particularly in stressed soils. Though economically important, sunflower crop is affected badly by several biotic and abiotic stresses. It has been estimated that among abiotic stresses salinity is the major constraint which limits sunflower yield. More than 60% loss in the production of sunflower is due to salinization stress in such soils (Khan 2007). Unfortunately sunflower is attacked with variety of fungal pathogens as well, which affect its yield and oil quality. *M. phaseolina* is the most destructive phytopathogen causing heavy loss to Indian economy. The soil borne fungus *M. phaseolina* is endemic to temperate and tropical regions of the world and has wide host range; it can infect more than 500 different plant species including sunflower (Sadashivaiah et al. 1986; Rasheed et al. 2004). *M. phaseolina* is a fungal opportunist that likes to take advantage of stressed sunflower plant which is cultivated in salinized regions and causes 70% reduction in its oil production yield (Ullah et al. 2011). The soil borne fungus causes charcoal rot of sunflower at seedling infection stage. If the invaded plant survives, the fungus moves to the above ground parts. Temperatures near 30°C and dry conditions are optimal for *M. phaseolina* growth, which makes this pathogen prevalent in semi-arid and arid regions of the world including India, Pakistan, China, Uruguay, Spain, Russia and USA (Tančić et al. 2012). Generally, it is estimated that charcoal rot affects the crop throughout the world reducing seed yields by 20-36 % (Tančić et al. 2012).

Chemical treatment of *M. phaseolina* is often uneconomical and unfeasible. Biological control of this plant pathogen provides solution that cannot or only partially be managed by other control strategies (Kaur et al. 2012). There is a genuine interest in developing plant growth promoting (PGP) and biocontrol products that are reliable and applicable for a number of crops, working under salinized soil.

Significant number of bacterial biocontrol products based on *Pseudomonas*, *Bacillus*, *Streptomyces* and *Agrobacterium* species have already been marketed and commercial preparations based on rhizobacteria are steadily increasing. Despite the fact that a number of *Pseudomonas* bioformulations are in the market there are still some limitations, hampering the development of this technology for wide-spread use in agriculture under salinized conditions. Although many strains show good performance in specific trials, this is often not translated into consistent, effective PGP and biocontrol inoculums under diverse conditions (Bashan et al. 2014). This is due to external factors such as soil or climatic conditions, but a major reason is the result of intrinsic traits of the microbes, such as variable production of required metabolites or poor colonization under stressed conditions.

Determination of active metabolites for plant growth promotion and biological control working under saline conditions are needed to be elucidated. Utilization of such metabolites with effective carriers in farmers' fields can provide effective solution. For all kinds of inoculants, development of multitasking bioformulations is one of the alternatives to overcome inconsistent *in vivo* effects. The microbial/ metabolites/ metabolite-microbial consortia that promote plant growth and suppress plant disease under saline conditions can be of great significance for sustainable agriculture production. Such products are replicable, reliable and having global applicability of enormous importance for end users.

The main objectives of the study include isolation and identification of saline tolerating fluorescent Pseudomonads, monitoring PGP and biocontrol potential of the isolates under saline conditions, to determine the strategy opted by microbes for surviving under saline stress and finally the development of cell / metabolite based bioformulations working under field conditions taking sunflower as a test crop (Figure 1).

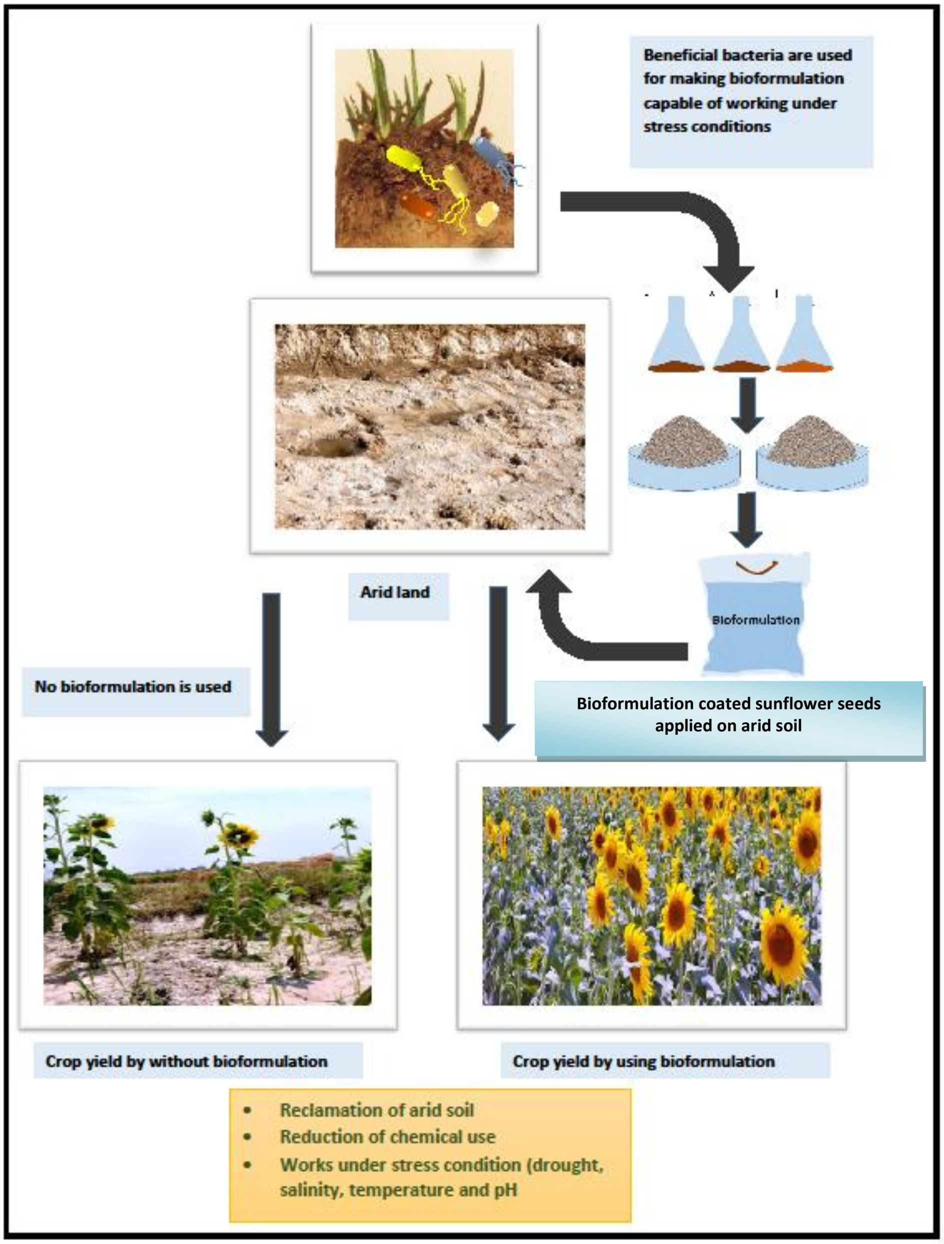


Figure 1: Increasing the productivity of sunflower in saline habitat utilizing bioformulations

Chapter 2

Literature Review

Food security is becoming a major and fast growing concern worldwide. It is proposed that there is a need to double the world food production in order to feed the ever increasing population which is set to reach nine billion mark by 2050 (Shaikh and Sayyed 2015). In the current scenario, improving crop yield in both normal and less productive farm lands including salt affected areas is one of the ways to address food security concern. Amongst various factors affecting agricultural production, abiotic stress factors are considered to be the main cause of yield reduction. Potential yield losses due to individual abiotic stresses are estimated at 17% by drought, 20% by salinity, 40% by high temperature, 15% by low temperature and 18% by other factors (Ashraf and Harris 2004). Till date, large amount of work has been done on drought and temperature stress but very little efforts are made to overcome the constraints of salinization stress (Hafeez et al. 2000; Lee 2009; Andre´s et al. 2012).

2.1 Salinization Stress

Soil salinity is one of the major abiotic stress factors affecting production and quality of food crops world-wide by limiting growth, development as well as yield potential of crop plants (Tester and Davenport 2003). More than 20% of the world arable land is now under the threat of salt stress. Soil salinity leads to accumulation of high concentration of sodium (Na^+), chloride (Cl^-) ions in plant cells, inactivating enzymes and inhibiting protein synthesis (Karmakar et al. 2015). Agricultural losses due to salinity in United States alone are estimated to be about US$12 billion a year, and are expected to rise as soils are expected to be further affected by salinity (Munns 2002; Munns and Tester 2008). Arid and semi-arid lands are more prone to salinity. In addition to primary salinization of seashores salty marshes, a significant portion of cultivated agricultural land is becoming saline due to deforestation, excess of irrigation and fertilization as well as poor drainage (Arora et al. 2012).

Soil salinization is increasing regularly throughout the world, and has become a great menace for agriculture. It has been estimated that about 10 million ha land in India is suffering from the problem of salinity and Uttar Pradesh alone has about 1.28 million ha saline lands (Kumar et al. 2014) (Figure 2). Salinity affects growth and production of diverse crops, including oilseed crops.

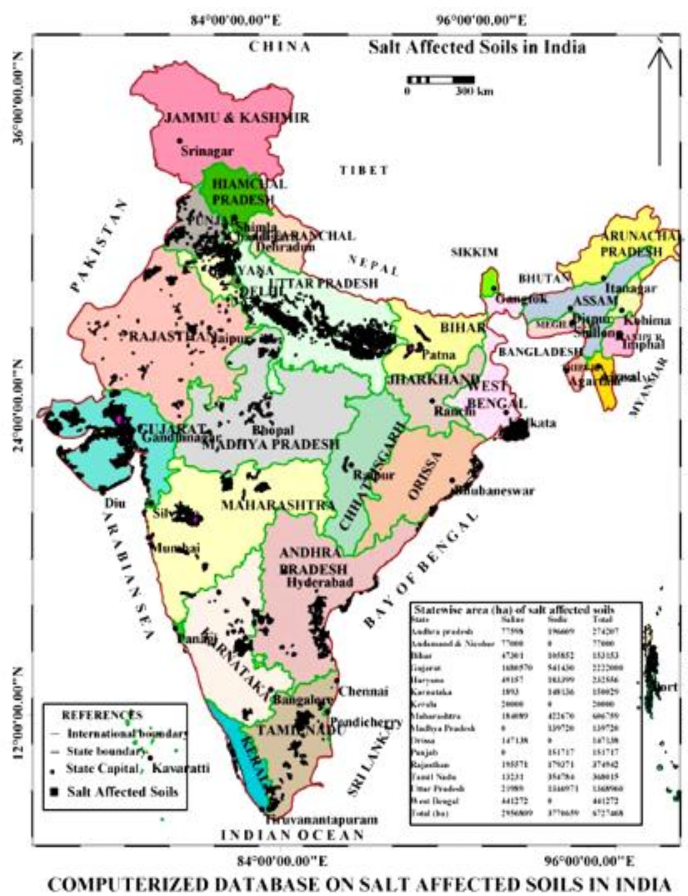


Figure 2: Saline lands of India (Source CSSRI Annual report 2011-2012)

2.2 Effect of Salinity on Oilseed Crop Sunflower

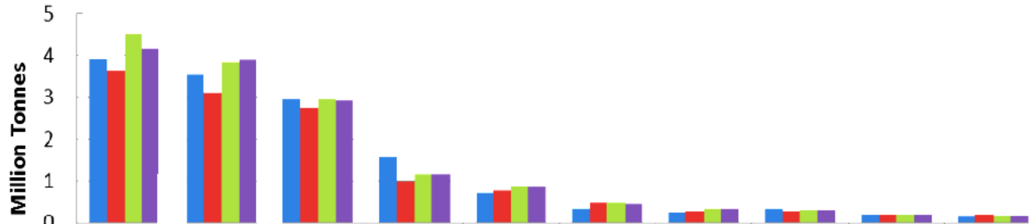
World's largest edible oil consuming countries are the USA, China, Brazil and India (Dubey and Maheshwari 2011). India contributes about 8% of the world oilseed production and about 6% of the global production of oils and fats, and currently is the fifth largest vegetable oil economy in the world, after the USA, China, Brazil and Argentina (Ramesh and Hegde 2010). Oilseeds contribute much towards attaining food security. A growing population, increasing rate of consumption and increasing per capita income are accelerating the demand for edible oil globally. Amongst diverse oilseed crops, sunflower commonly known as '*Surajmukhi*' is one of the most potential oil yielding crop gaining popularity because of its wider adaptability to different agroclimatic conditions and ample of health and dietary benefits (Dubey and Maheshwari 2011). Sunflower, an Asteraceae (Compositeae) family plant, is native to the temperate North America, which is the center of diversity for this important edible oil-yielding species. It is a short duration crop which is adaptable to a wide range of agroclimatic situations, having high yield potential, suitable for cultivation in all seasons due to its day neutral nature and can fit well in various inter and sequence cropping systems (Yadava et al. 2012). Sunflower is grown in all continents. Europe and America account for nearly 70% of total area and 80% of total production (Damodaran and Hegde 2005). Its cultivation in Asian countries is comparatively recent. Asia accounts for nearly 20–22% of the global area under sunflower cultivation and contributes to about 18% of the production (Yadava et al. 2012). Emergence of new diseases and climatic variations, particularly occurrence of saline stress during critical growth stage has affected stability and yield on a regular basis.

Abiotic and biotic factors play key role in lowering the productivity of sunflower crop at different stages of crop growth that is an alarming feature (Ranasingh and Mahalik 2008). Salt stress affects both vegetative and reproductive growth stages by reducing growth, delaying the onset of flowering, reducing the quantum of reproductive structures and delaying maturation processes, ultimately leading to diminished crop yields (Figure 3). Additionally, it affects production of several enzymes leading to the reduction of glycerides and modification of fatty acid profile, important quality determinants of seed oil. To avoid these losses, it is essential to take biological measures for enhancing crop growth and productivity. The need for sustainable resource management utilizing biological approaches is increasingly urgent. The demand for agricultural commodities is rising rapidly as the world's population grows. Agriculture has deep connections to the world economy, human societies and biodiversity making it one of the most important frontiers for conservation around the globe (Sharma and Kaur 2003).

Figure 3 : Effect of salinity on sunflower crop

2.3 Sunflower Production in India

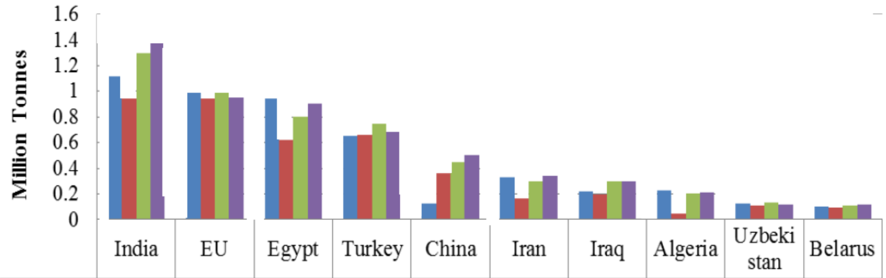
Sunflower production in India as an edible oilseed crop took-off commercially from 1972 onwards. Its acreage has expanded remarkably from about 500 ha in the early 1970s to about 2 million ha in the early 1990s (Berglund and Duane 2009). The expansion in area continued till mid-2000s except for few years during 1999 to 2003. Oilseeds are among the major crops that are grown in this country apart from cereals. Ukraine is the largest producer of sunflower in the world followed by Russia and European Union (EU) in 2014-15. However, India ranks 9th in this race (Figure 4).



	Ukraine	Russia	EU	Argentin a	Turkey	China	South Africa	Pakistan	India	United States
2011/12	3.933	3.552	2.937	1.565	0.718	0.348	0.272	0.34	0.205	0.149
2012/13	3.615	3.117	2.753	0.99	0.769	0.476	0.278	0.285	0.22	0.196
2013/14	4.515	3.851	2.937	1.16	0.875	0.49	0.335	0.313	0.21	0.181
2014/15	4.175	3.92	2.926	1.18	0.867	0.455	0.335	0.33	0.2	0.172

Figure 4 : Production trend of sunflower (Commodity profile 2015)

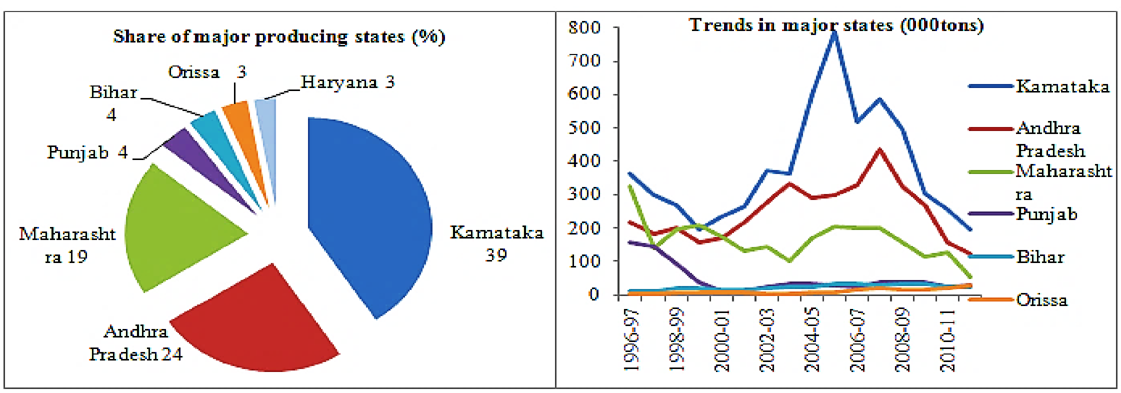
India's share in global production of sunflower oil was 1.37 percent in the year 2014-15. Ukraine and Russia were found to be the top two global exporters of sunflower oil in 2014-15. India is the largest importer of Sunflower oil followed by EU, Egypt, and Turkey during 2014-15 (Figure 5) (Commodity profile 2015). Sunflower oil market in India has been growing magnificently owing to high growth of income levels, increasing trend towards spending, better living standards and growing health consciousness among Indian consumers. In the year 2011-12, sunflower oil market in India was more than Rs 150 billion which is expected to grow with a compound annual growth rate of around 9.5% during 2013-14 to 2016-17.



	India	EU	Egypt	Turkey	China	Iran	Iraq	Algeria	Uzbekistan	Belarus
2011/12	1.114	0.991	0.94	0.651	0.122	0.333	0.216	0.229	0.123	0.098
2012/13	0.939	0.942	0.62	0.657	0.362	0.163	0.197	0.048	0.111	0.097
2013/14	1.3	1	0.8	0.75	0.45	0.3	0.3	0.2	0.13	0.11
2014/15	1.4	0.95	0.9	0.68	0.5	0.35	0.3	0.21	0.12	0.115

Figure 5: Major sunflower importing countries in the world (Commodity profile 2015)

Oilseed sector occupies an important position in the agricultural economy of India. In India, Karnataka is the leading producer of sunflower followed by Andhra Pradesh and Maharashtra, whereas Uttar Pradesh lags far behind due to its high semi-arid habitat (GOI 2004) (Figure 6).



Source: Ministry of Agriculture, GoI

Figure 6: Major sunflower producing states in India (GOI 2004)

Amongst various states, Karnataka, Andhra Pradesh and Maharashtra together contribute for about 83 % of total production in the country. Area and output from all the major states followed a similar pattern of rise in production until mid-2000 and declined thereafter. However, production in Punjab and Haryana has declined consistently during the past 15 years (Semerci et al. 2011). The area and production of sunflower in India has started declining consistently from 2005 onwards. As a result, India slipped from earlier sixth to ninth position in the world sunflower output (commodities profile 2015). Several studies reported that nowadays, production of sunflower is affected badly by several abiotic and biotic stresses (Katerji et al. 1996; Mohammed et al. 2002; Muhammad et al. 2010; Shilev et al. 2010; Tewari and Arora 2014b). Abiotic stresses include the constraints of salinization stress (Mostafavi and Heidarian 2012) whereas biotic stress include the effect of dreadful phytopathogen *M. phaseolina* (Khan 2007; Ullah et al. 2011).

2.4 *Macrophomina phaseolina*

M. phaseolina (Tassi) Goid. (*Tiarosporella phaseolina* (Tassi) Vander Aa) is a soilborne plant pathogenic fungus. *M. phaseolina* is an anamorphic fungus in the ascomycete family Botryosphaeriaceae. It is characterized by the production of both pycnidia and sclerotia in host tissues and culture media. The pycnidial state was initially named *Macrophoma phaseolina* by Tassi in 1901 and *Macrophoma phaseoli* by Maublanc in 1905. In 1927, Ashby maintained the name *Macrophomina phaseoli*, while Goidanich in (1947) proposed *Macrophomina phaseolina*. *Tiarosporella phaseolina* (Tassi) Van der Aa was used in 1981 by Van der Aa to designate the species (Goidanich 1947). Mihail (1992) indicated that there is an unconfirmed report of a teleomorph named *Orbilia obscura* of *M. phaseolina*, but since then no further evidence appeared for the telemorph state. The sclerotial state was described for the first time by Halsted as *Rhizoctonia bataticola* (Taub.). According to Dhingra and Sinclair (1978), the same fungus was isolated from cowpea in India in 1912 by Shaw and was then named *Sclerotium bataticola*.

M. phaseolina is one of the most destructive necrotrophic fungal pathogens that infect more than 500 plant species throughout the world. It can grow rapidly in infected plants and subsequently produces a large amount of sclerotia that plugs the vessels, resulting in wilting of the plant (Islam et al. 2012). It causes stem canker, seedling blight, charcoal rot, dry root rot, wilt, leaf blight; stem blight, pre-emergence and post-emergence damping-off (Singh et al. 1990), root and stem rot of softwood forest trees (Mc Cain and Scharpf 1989), fruit trees and weed species (Songa and Hillocks 1996) (Figure 7). The fungus has a vast geographical distribution and is especially problematic in tropical and subtropical countries with arid to semiarid climates in Africa, Asia, Europe, North and South America (Gray et al. 1990; Diourte et al. 1995).

Figure 7: Losses in sunflower production due to *M. phaseolina*

M. phaseolina is primarily soil borne in nature, with heterogeneous host specificity, that is, the ability to infect monocots as well as dicots and non-uniform distribution in the soil (Mayek-Perez et al. 2001). The pathogen is also seed-borne and seed-to-seedling transmission has been documented in infected seeds of sunflower plant. *M. phaseolina* infection on sunflower was first reported from Sri Lanka in 1927. Subsequently, it was reported from Uruguay, Australia and Yugoslavia in 1966, Argentina (1967), Hungary (1970), USA (1971), India (1973), France (1976), Egypt (1980) and Pakistan, (1982) (Khan 2007). *M. phaseolina* is a serious threat for sunflower crop especially in the arid regions of the world (Hoes 1985). Yield losses claimed by charcoal rot in Spain, United States, Uruguay and Soviet Union have been recorded upto 25%, however, under favorable conditions growth and development of *M. phaseolina* causes total failure of crop in specific regions (Jimenez et al. 1983). Charcoal rot is of great economic importance in arid areas of the world as it causes decrease in stem height, girth, root and head weight in sunflower (Khan 2007). Under favorable conditions 90 % prevalence of charcoal rot with severe intensity has been reported from major sunflower growing areas of Punjab in India (Rana 1999). Climatic conditions like temperature, salinity, atmospheric humidity, and available moisture play a significant role in activation and multiplication of *Macrophomina*. The pathogen takes advantage of stress and dry conditions and causes severe damage in sunflower crop (Khan 2007). Up to 60% yield losses due to charcoal rot in certain regions have been reported by Steven et al. (1987).

Chemical amelioration has been tried to overcome the destruction caused by phytopathogen and losses due to salinization, but the use of pesticides has caused an incredible harm to the environment. These agents are both hazardous to animals and humans and may persist and accumulate in natural ecosystems. An alternative solution to this problem is replacing chemicals with biological approaches (such as PGPRs), which are considered more environment friendly in the long term.

2.5 Plant Growth Promoting Rhizobacteria

The term PGPR was first used by Kloepper and Schroth (1978) to describe the bacteria that colonize the plant roots. These microorganisms generally exist more or less near the roots, due to the presence of root exudates that are the source of food for their growth (Whipps 1990) and most of them depend on root exudates for their survival (Glick 1995; Khalid et al. 2006). Most of these

PGPRs belong to genera including *Serratia*, *Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Bacillus*, *Azoarcus*, *Paenibacillus*, *Klebsiella*, *Erwinia*, *Beijerinckia*, *Flavobacterium* and *Gluconacetobacter* (Okon and Labandera-Gonzalez 1994; Glick 1995; Podile and Kishore 2006; Arora et al. 2012).

Some researchers have described PGPR on the basis of their growth promoting characteristics. For example, Bashan and Holguin (1998) categorized the PGPR into two classes i.e. biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. Khan (2005) on the basis of relationship of rhizobacteria with plants divided them into two groups i.e. symbiotic rhizobacteria and free-living rhizobacteria. However, Gray and Smith (2005) classified PGPR as extracellular and intracellular. According to their view, extracellular PGPR exist in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex while intracellular PGPR exist inside the root cells. Sayyed et al. (2010) termed beneficial microorganisms as yield increasing bacteria (YIB). Burr and Caesar (1984) reported these bacteria as plant health promoting rhizobacteria (PHPR) and nodule promoting rhizobacteria that are present in the rhizosphere. In view of Vessey (2003), PGPR are bacteria which flourish in the rhizosphere and may grow in, on, or around plant tissues and enhance growth and development. Gray and Smith (2005) mentioned the gradient of root proximity among rhizobacteria which is: bacteria living near roots, bacteria in the rhizoplane, bacteria within the root tissues and the bacteria that reside inside the cell in specialized structures called nodule.

There are number of ways through which PGPR are helpful for promoting plant growth and development. According to Glick (1995), PGPR facilitate plant growth by three different ways that include the synthesis of compounds for plant uptake, facilitating the nutrient uptake and disease prevention (Garcia de Salamone et al. 2001; Raj et al. 2003; Guo et al. 2004). PGPR use various mechanisms which may take place simultaneously or sequentially at different plant growth stages.

These mechanisms are divided in to two major categories i.e. direct and indirect growth promoting mechanisms (Scher and Baker 1982; Shanahan et al. 1992; Glick 1995; Boddey and Dobereiner 1995; Flaishman et al. 1996). Phosphate solubilization, phytohormones and siderophores production are some examples of direct growth promotion by the PGPR (Kloepper et al. 1989; Glick 1995). PGPR also play important role indirectly by inhibiting the growth of plant pathogens (Glick and Bashan 1997; Persello-Cartieaux et al. 2003). This can be achieved by the production of antibiotics and antifungal metabolites, lytic enzymes, hydrogen cyanide, inducing systemic resistance and also by reducing the availability of certain nutrients (such as iron) required by the pathogen for its growth (Van Loon 2007; Labuschagne et al. 2010; Pandya and Saraf 2015). Arora et al. (2013) reported that these so-called direct and indirect mechanisms, however, hold well in theoretical and literal explanation only. In nature, all these mechanisms are interconnected and interwoven. In natural conditions the compounds or metabolites released by microbes function in multifaceted and diverse manners. A single compound released by microbes can play diverse functions depending upon the conditions or may perform similar functions under diverse conditions depending upon the biotic and abiotic factors. Several PGPR strains of *Pseudomonas* show both direct and indirect mechanisms to enhance plant growth as well as act as biocontrol agents (Selvakumar et al. 2015). In addition to these general mechanisms of growth promotion, PGPR also protect the plant from the deleterious effects of various environmental stresses (Brahim et al. 2015). Stress conditions, not only affect the plant growth but are also harmful for the beneficial microbial community. Hence the need is to hunt for those PGPRs which maintain their growth under stress conditions.

2.6 Fluorescent Pseudomonads

The genus *Pseudomonas* (derived from the Greek words *pseudes* "false" and *monas* "a single unit" or "false unit"), comprises one of the most diverse and ecologically fit groups of bacteria on this planet, whose members are collectively referred by the generic term Pseudomonad's. Taxonomically the genus *Pseudomonas* falls within the γ subclass of Proteobacteria, and its ubiquity can be gauged by its ability to colonize terrestrial, freshwater and marine environments with relative ease, besides forming intimate associations with higher forms of life (Selvakumar et al. 2015).

The genus *Pseudomonas* was first described by Migula (1894) and has been a part of the history of Bacteriology ever since its inception. Though initially *Pseudomonas* was described as a genus of Gram-negative, rod-shaped and polar-flagella bacteria with some sporulating species, it was later proved that the so called spores were actually refractive granules of reserve materials. The physiological diversity of this bacterial genus was unravelled by the pioneering studies of bacteriologists such as Beijerinck (1921) and Winogradsky (1949) during the first half of the 20th century. The 1923 edition of the Bergey's Manual (Bergey et al.1923) included a chapter on the genus *Pseudomonas*, where the classification of species was based on phenotypic characteristics. But the initial classification of *Pseudomonas* as Gram negative, aerobic non-sporulated rods that are motile by means of polar flagella did not differentiate it sufficiently from other Gram negative bacteria, and led to the dumping of several improperly characterized Gram negative bacteria. But later day observations that incorporated other phenotypic characteristics were able to bring about a degree of clarity on the taxonomical boundaries of this genus. A monumental work in the 1960s, to clarify the taxonomy of the genus was carried out by Stanier et al. (1966). This exhaustive and highly cited work which reported the nutritional characteristics of 267 strains on 146 different organic compounds plus a wide range of associated characteristics is considered as a classic study on bacterial taxonomy (Spiers et al. 2000). The development of DNA- DNA hybridization methodologies, during the same decade led to the confirmation and reclassification of several phenotypic data, which ultimately led to the inclusion of the G+C content of bacteria in the Bergeys Manual edition published in 1974, which included 29 species (Doudoroff and Palleroni 1974). During this period another development in classification of genus *Pseudomonas* by genotypic criteria was made by Palleroni and co-workers, who classified this bacterial group into five rRNA subgroups based on the measurements of RNA–DNA relatedness (Palleroni et al. 1973). These rRNA subdivisions were phylogenetically very distant, and only the representatives included in the rRNA group I were finally included in genus *Pseudomonas*, while the members of the other subgroups were reclassified as separate genera. Though this approach failed to find a place in the 1974 edition of the Bergey’s Manual, it was accommodated in the 1984 edition of the Bergey’s Manual, with minor modifications in the number of species (Palleroni 1984). The 1980s saw the emergence of bacterial taxonomy based on the sequencing of the 16S rRNA gene (Woese et al. 1984). But this scheme of classification was not reflected in the Bergey’s Manual of Determinative Bacteriology published in 1994, where species determination was carried solely on the basis of phenotypic characters. This edition included only the classical species of *Pseudomonas* while the newly described species, were included as an additional list. The current edition of Bergey’s Manual of Systematic Bacteriology, contains an exhaustive review of the criteria used in the modern taxonomy of genus *Pseudomonas* and includes fifty nine species that were described till the year 2005 (Palleroni 2005).

Fluorescent *Pseudomonas* group includes (1) phtopathogenic cytochrome c oxidase – positive species, viz. *P. cichorii*, *P. marginalis* and *P. tolaasii*, (2) non-phytopathogenic, non-necrogenic strains viz. *P. fluorescence*, *P. putida*, *P. chlororaphis*, *P. aureofaciens* and *P. aeruginosa*, (3) phytopathogenic necrogenic fluorescent *Pseudomonas* spp. without cytochrome c oxidase, viz. *P.*

syringae and *P. viridiflava.* The non-fluorescent *Pseudomonas* group includes *P. stutzeri*, *P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes* (Palleroni et al. 1973).

Fluorescent Pseudomonads help in the maintenance of soil health, protect crops from pathogens, promote plant growth and are metabolically and functionally most diverse. *Pseudomonas* spp. are ubiquitous in agricultural soils and are well adapted to growing in the rhizosphere. Pseudomonads possess many traits that make them well suited as biocontrol and growth promting agents (Weller 1998). These include ability to (i) grow rapidly *in vitro* and to be mass produced; (ii) rapidly utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and spermosphere environment and in the interior of the plant; (iv) produce a wide spectrum of bioactive metabolites (i.e. antibitiotics, siderophores, volatiles and growth promoting substances); (v) compete aggressively with other microbes; (vi) adapt to environmental stress; (vii) subsequently be reintroduced onto the rhizosphere by seed bacterization and suspectible to mutations using state of the art genetic tools (Lughtenberg et al. 1994).

Pseudomonas sp. have been reported to with stand salinity stress by diverse mechanisms including the production of exopolysaccharides (EPS). These EPS producing microbes are well used in enhancing growth parameters and in antagonizing dreadful phytopathogens (Tewari and Arora 2014a). Under stress conditions PGPR work through particular mechanisms like lowering of stress-induced ethylene, production of EPS, regulating nutrient uptake, enhancing the activity of antioxidant enzymes, producing salicylic acid (SA) and suppressing the growth of pathogens (Sandhya et al. 2010; Glick 2012; Nadeem et al. 2012; Saharan and Nehra 2011). EPS and SA producing microorganisms are imparting salt tolerance to bacterial cells but relatively little attention has been paid on their utility and role under saline conditions (Potts 1994). Diverse PGPRs and their mechanistic metabolites could be utilized for developing multifaceted bioformulations working under saline conditions for enhancing plant growth and yields.

2.7 Bioformulations for Sustainable Agriculture

Bioformulations are best defined as biologically active products containing one or more beneficial microbial strains in easy to use and economical carrier materials. Usually, the term bioformulation refers to preparations of microorganism(s) that may be a partial or complete substitute for chemical fertilizers/pesticides (Arora et al. 2010). These formulations are carrier based preparations containing beneficial microorganisms in viable state intended for seed or soil application, which enhances plant growth through nutrient uptake or growth hormones production. The commercial history of bioformualtion with biofertilizer activity began with the launch of Nitragin in 1895 by two German scientist Nobbe and Hiltner, who demonstrated the advantage of adding pure bacteria with the seed. The first patent (Nitragin) was registered for plant inoculation with *Rhizobium* sp. about a century ago (Nobbe and Hiltner 1896). Different countries have launched *Azospirillum* as a biofertilizer for different crops. Azo-Green™, Zea-Nit™ and Graminante™ are some of the commercialized products manufactured from these bacteria (Mehnaz 2015). Pseudomonads are also being investigated extensively for use in plant growth promotion and biocontrol of pathogens in agriculture (Ganeshan and Kumar 2006). They are known to enhance plant growth, yield, reduce severity of many diseases and are considered to be amongst the most prolific PGPRs (Wei et al.1996). Several species of *Pseudomonas* are being used for designing bioproducts that include *P. fluorescence*, *P. aeruginosa*, *P. syringae* etc. Certain strains of *Pseudomonas aureofaciens* are being used against a range of plant pathogens including damping-off and soft rots (Kloepper et al. 2004; Haas and De'fago 2005). The cell suspensions of Pseudomonads are immobilized on certain carriers and are prepared as formulations for easy application, storage, commercialization and field use. In India *P. fluorescens* biopesticide is effectively being used against late blight of potato; it is available commercially under diverse brand names such as Krishi bio rahat, Krishi bio nidan, Mona etc. Virulent cells of bacterial antagonist *P. fluorescens* are taken to prepare a biopesticide formulation that is effective against phytopathogen *Ralstonia solanacearum* (Chakravarty and Kalita 2011).

Attractive role of fluorescent Pseudomonads in biological control of fungal plant pathogens has been illustrated against *Aspergillus, Alternaria, Fusarium, Macrophomina, Pythium, Sclerotinia* and *Rhizoctonia* (Gupta et al. 2001). Several different commercially available bioformulations in US that are developed from *Pseudomonas* are Spot-Less, At-Eze, Biosave 10LP, Bio-Save 11LP (Vargas 1999; Nakkeeran et al. 2005; Khalil et al. 2013). Development of formulation from fluorescent Pseudomonads serving multifaceted functions of plant growth promotion, bioremediation and disease management is the dire need of the day. However, the erratic performances of bioformualtions under field conditions have raised concerns about the practical potential offered by microbial releases into soil (Arora et al. 2007; Mishra et al. 2015). Although much is known about the survival of bacteria within the protective environment of an inoculant carrier, little is known about the stresses that bacteria must endure upon transfer to the competitive and often harsh soil environment (Heijnen et al. 1992).

Bioformulations have to be designed to provide a dependable source of beneficial bacteria that survive in the soil and become available to the plant. Use of stress-tolerating strains of PGPR, there biopreparations either as aqueous suspensions or as bioformulations of sawdust, rice husk, tea waste, and talc-based bioformulants promoting growth in agricultural crops even under saline conditions is a demanding task (Ross et al. 2000). The development of novel formulations is a challenging work but, regardless of whether the product is new or improved, it must be stable during storage and transportation, easy to handle and apply, enhance the activity of the organism in the field, and be cost-effective (Young et al. 2006). Although PGPR inoculation has been proved useful for enhancing plant growth and development in normal as well as in saline stress conditions, in certain cases particularly in field, inconsistent results are obtained with single inoculum (Bashan et al. 2014; Vassilev et al. 2015). This inability of bacteria to show their full potential might be due to certain factors including competition with indigenous population and low quality inoculum. The use of dual or multi-strain inoculum or its metabolites could be one of the best solutions to this problem. The cell free metabolite (CFM) based consortium of beneficial bacteria, working together under a common regulon can give significant output. Metabolites of fluorescent Pseudomonads play key role in growth promotion and suppression of various soil-borne plant pathogens. The use of these metabolites for development of bioformulation is unique and novel approach towards reliable and sustainable agriculture production.

Thus developing cell free metabolite based bioformulation (CFMB) from Pseudomonads and checking its applicability for different agro-climatic zones, diverse soil types and crops will therefore be of tremendous liking to produce more and more food from shrinking per capita arable land while keeping the environment safe.

Soil being a highly heterogeneous and unpredictable environment with complex microcosm the inoculant microbes do not find conditions to their liking, which are provided to them in the laboratory. Isolation, screening and identification of PGPRs with higher tolerance to edaphic factors including temperature extreme, suboptimal salinity and variation in pH could be effectively utilized for enhancing yield of diverse crops under saline conditions.

In arid and semiarid areas harsh conditions, including frequent droughts, lack of sufficient irrigation, high salinity and soil erosion leads to development of unfavorable agriculture conditions.

Reclamation of such areas utilizing beneficial microorganism(s) will make good contribution to the Indian economy, if inexpensive and easy to use stress tolerant strain-formulation(s) can be developed (Figure 8).

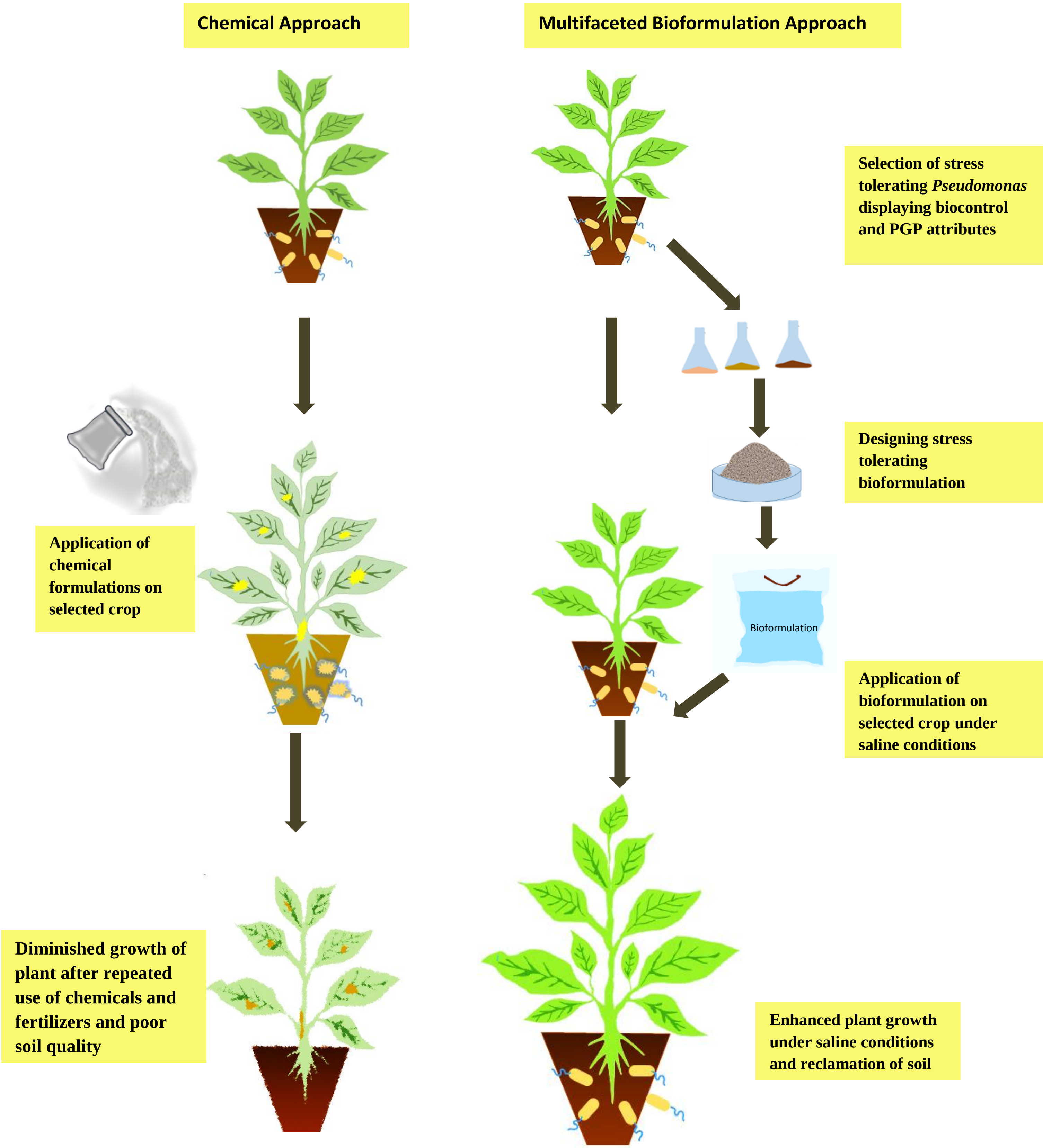


Figure 8: Development of multifaceted bioformulation working under saline conditions

Chapter 3

Materials
&
Methods

3.1 Bacterial Strains

In total 110 fluorescent bacteria were isolated from the rhizosphere of different plants growing in the semi-arid regions of Kanpur Dehat (20°38′ E and 80° 21′ N; temperature maximum 48°C and minimum 1°C), Uttar Pradesh (northern India) (Table 1). The area comes under the Gangetic plains. The soil samples were collected from the root surface, serially diluted to 10^{-6} in distilled water (DW), plated on King's B medium (KB) containing (g^{-1}) in DW: protease peptone, 20; K_2HPO_4, 1.5; $MgSO_4$, 1.5; agar-agar 1.5g; (King et al. 1954) and the plates were incubated at 28°C for 24 h. Colonies that fluoresced under UV light (GENEI UV-Transilluminator, Model MD-20) were selected and further purified on the same medium. The isolates were grown on KB agar at 28°C and maintained in 25% glycerol at – 40°C (Remi Quick Freezer, Model RQF-170).

3.2 Phenotypic Characters

Morphology of the isolates was checked by simple staining and Gram- staining. Motility was determined by observing a drop of culture under oil immersion of microscope.

3.3 Biochemical and Physiological Characters

The isolates were further tested for their physiological and biochemical characters according to the procedure outlined by Cappuccino and Sherman (2005) and identified by referring to Bergey's Manual of Systematic Bacteriology (Garrity 2005). Briefly isolates were checked for catalase, oxidase activity, production of acid from glucose and glycerol, starch hydrolysis, gelatin hydrolysis, citrate utilization, fluorescein and pyocyanin production. Growth of the isolates in presence of 2% NaCl and 8% KNO_3 was also checked by spectrophotometric analysis.

Table 1 : Different isolates obtained from the rhizosphere of diverse plants

S.No	Name of plant	Number of isolates	Isolates	S. No	Name of plant	Number of isolates	Isolates
1.	Amla (*Emblica officinalis*)	01	AM1	17.	Mango (*Magnifera indica*)	05	MA1-MA5
2.	Anar (*Punica granatum*)	01	AN2	18.	Marigold (*Tagetes erecta*)	04	MR1- MR4
3.	Ashok (*Saraca indica*)	03	AS1- AS3	19.	Medicinal Aloe (*Aloe vera*)	09	AL1-AL9
4.	Bamboo (*Phyllostachys aurea*)	02	BA1- BA2	20.	Methi (*Trigonella foenum-graecum*)	04	MT1-MT4
5.	Bathua (*Chenopodium album*)	01	BT1	21.	Mulbery (*Morus nigra*)	05	ML1-ML5
6.	Ber (*Ziziphus* mauritiana)	01	BE1	22.	Mustard (*Brassica Campestris*)	06	MU1-MU6
7.	Black gram (*Vigna mungo*)	02	BG1- BG2	23.	Neem (*Azadirachata indica*)	02	NE1-NE2
8.	Castor oil plant (*Ricinus communis*)	02	CA1-CA2	24.	Papaya (*Carica papaya*)	02	PA1-PA2
9.	Chickpea (*Cicer arietinum*)	04	CH1- CH4	25.	Potato (*Soloanum tuberosum*)	07	PO1-PO7
10.	Cotton (*Gossipium sp.*)	02	CO1, CO2	26.	Pudina (*Mentha arvensis*)	02	PU1
11.	Garlic (*Allium sativum*)	03	GA1- GA3	27.	Rose (*Rosa L.)*	01	RO1
12.	Ginger (*Zingiber officinale*)	01	GI1,	28.	Sadabahar (*Vinca rosea*)	01	SD1
13.	Groundnut (*Arachis hypogea*)	04	GN1- GN4	29.	Sunflower (*Helianthus annuus*)	16	PF07-PF23
14.	Guava (*Psidium guajava*)	02	GU1- GU2	30.	Tamarind (*Tamarindus indica*)	02	TA1-TA2
15.	Guldaudi (*Dahlia sp.*)	03	GL1-GL3	31.	Tomato (*Lycopersicon esculentum*)	04	TO1-TO4
16.	Gurhal (*Hibiscus rosa-sinensis*)	02	GR1, GR2	32.	Tulsi (*Ocimum sanctum*)	06	TL1-TL6

Total 110 isolates

3.3.1 Growth Kinetics

Growth of isolates with different colony characters (small, transparent, slightly fluorescent colonies; large fluorescent colonies with diffuse margin; reddish brown pigmentation on storage at 4°C) was checked up to 24 h in KB broth after every 2 h interval by spectrophotometer (Thermofisher Scientific Evolution 201). Optical density (OD) was checked at 610 nm and generation time was calculated as: Generation time (g) = t/n Where, t = hours of exponential growth, n (number of generations) = log N – log N_0 / 0.301; N and N_0 = two different Klett values (OD/0.002) taken between a time interval (t).

3.3.2 Biolog Analysis

Pseudomonas isolates were subjected to carbon source utilization pattern studies using Biolog GN2 micro plate. Briefly, pure culture of bacterium was grown on KB agar plate. The bacteria were swabbed from the surface of the agar plate, and suspended to a specified density in Gram negative inoculating fluid. 150 µl of bacterial suspension was pipetted into each well of the GN2 micro plate. The micro plate was incubated at 28ºC for 24 h and absorbance was taken at 590 nm with plate reader (Tang et al. 1998).

3.3.3 Intrinsic Antibiotic Resistance Test

The antibiotic resistance test of the isolates was done for ampikacin, amoxicillin, ampicillin, cephalothin, erythromycin, bacitracin, ciprofloxacin, chloramphenicol, cloxallin, cephradine, cephatoxamine, ceftezidine, carbenicillin, cephoxitin, clindamycin, cefoperazone, erythromidazole, furazolidone, gentamycin, lincomycin, metronidazole, novobiocin, nitrofurantoin, nalidixic acid, norfloxacin, neomycin, oflacin, oxytetracyclin, penicillin G, piperacillin, penicillin P, rifampicin, streptomycin, tetracycline, tobramycin and ticarcilline, at 25 μg/ml concentration by Kirby-Bauer method or Disc diffusion method (Bauer et al. 1966).

3.3.4 Stress Tolerance Assay

The log phase culture (OD 610 = 0.1) of the isolates were seeded in Davis Minimal Media (DMM) broth [containing (g/l in distilled water) glucose, 4.0; $MgSO_4.7H_2O$, 0.1; K_2HPO_4, 7.0; $(NH_4)_2SO_4$, 1.0; Na-citrate, 0.5; and 1 ml of a trace element solution (MB medium). The trace element solution contained (mg/l): [$CoCl_2.6H_2O$, 20; H_3BO_3, 30; $ZnSO_4.H_2O$, 10; $CuCl_2.2H_2O$, 1; $NiCl_2.6H_2O$, 2; $NaMoO_4.2H_2O$, 3; $FeSo_4.7H_2O$, 10; $MnSO_4.H_2O$, 2.6] amended with different concentrations of NaCl (0 to 2,200 mM), temperature ranges from 10ºC to 60ºC and pH from 3 to 13. OD was measured at 610 nm by spectrophotometer (Thermofisher Scientific Evolution 201) up to stationary phase (120 rpm). The experiment was conducted in five replicates. Only two strains PF17 and PF23 displayed high salinity, temperature and pH tolerance. Hence, further studies were performed taking these two isolates.

Multistress tolerance assay was conducted on the basis of the results obtained from monostress studies. Upper limit of saline, temperature and pH tolerance of each variable was determined (depending upon the isolates) and on the basis of it, multistress tolerance assay was conducted. Upper limits of saline and alkaline pH were selected because in natural soil conditions salinization and alkalization are the main issues needed to be tackled.

Screening for the combined effects of temperature, NaCl and pH was done, by incubating the liquid culture with PF23 with diverse salt concentrations (0-2000 mM NaCl), adjusted at pH 12 and incubated at 45°C. Whereas, for PF17 medium was amended with 0-1400 mM NaCl adjusted with pH 13 and incubated at 50°C.

3.4 Plant Growth Promoting and Biocontrol Traits

3.4.1 Phosphate Solubilization

Phosphate solubilizing activity of the isolates was tested by spot inoculation on Pikovskaya's medium (PVK) amended with different concentrations of salt ranging from 0 to 2000 mM NaCl (Subba-Rao 1999). Log phase culture of all the isolates (0.5 µl) were spot inoculated on PVK agar (Pikovskaya 1948) plates and incubated for seven days at 28ºC to calculate solubilization index (SI).

Quantitative estimation of tricalcium phosphate (TCP) solubilization was conducted through broth assays at 28°C in Erlenmeyer flasks (250 ml) containing 100 ml of PKV broth (supplemented with different salt concentration). The broth was inoculated with log phase culture of the isolates. Autoclaved uninoculated medium served as control in each case. Each experiment was done in triplicate. Growth medium was withdrawn aseptically at 3 days interval from each flask (in duplicate) and filtered through Whatman no. 42 filter paper. The filtrate was assayed for P_2O_5 content by chlorostannous reduced molybdo-phosphoric acid blue colour method using spectrophotometer (Pandey et al. 2008).

3.4.2 Indole -3- Acetic Acid (IAA)

The production of IAA was determined according to the method of Bric et al. (1991). In brief, the isolates were inoculated in 5 ml KB broth (amended with 0 - 2000 mM NaCl), supplemented with tryptophan (500 µl/ml), following incubation at 28°C for 48 h and bacterial cells were removed from the culture media by centrifugation at 7826 × g for 15 min. An aliquot of 2 ml supernatant was transferred to a fresh tube to which 100 µl of 10 mM orthophosphoric acid and 4 ml of reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 min and absorbance was read at 530 nm. The concentration of IAA was evaluated by comparison with standard curve.

3.4.3 Siderophore

Qualitative production of siderophore by the selected isolates was determined on chrome-azurol S (CAS) medium supplemented with diverse salt concentrations (0-2000 mM NaCl). CAS plates were spot inoculated with bacterial isolates and observed for development of orange halo against dark blue background around the colonies after 48 h of incubation at 28°C (Schwyn and Neilands 1987).

For quantitative estimation the selected isolates were grown on KB broth (at different salinity levels) for 48 h at 28°C. After that culture broth was centrifuged (7826 × g for 15 min) and cell free supernatant was taken for detection and estimation of siderophore Payne 1994). In brief the method followed was 0.5 ml of CFCS was mixed with 0.5 ml of CAS reagent and absorbance was measured at 630 nm against the reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. Siderophore content in the aliquot were calculated according to the formula:

% siderophore units = Ar-As/ Ar x 100

Where, Ar = Absorbance of reference at 630 nm (CAS reagent)

As = Absorbance of sample at 630 nm

3.4.4 Hydroxymate Siderophore

Isolates were cultured in CAS broth (at different salt concentration) for 48 h at 28°C and centrifuged for 15 min to separate cells followed by membrane filteration (0.45µm, Merck India). CFCS was used to quantify hydroxymate type siderophore using absorption maximum and the molar absorption coefficient (λ max = 400 nm and ε 20,000 M^{-1} cm^{-1}) (Meyer and Abdallah 1978).

3.4.5 Catecholate Siderophore

To determine catechol production, Arnow's method was used (Arnow 1937). The isolates were grown in the respective media at diverse salinity for 48h. The assay was performed by mixing the following 1 ml culture supernatant, 1 ml 0.5 M HCl, 1 ml nitrite- molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml DW) and 1 ml 1M NaOH. These were allowed to incubate for 5 min for the reaction to fully occur. Absorbance was measured at 500 nm with uninoculated media serving as the blank and compared with standard curve of catechol.

3.4.6. Salicylate (Salicylic Acid) Siderophore

Qualitative and quantitative analysis of SA production by the isolates was done according to the method described by Meyer and Hofte (1997). Qualitative SA analysis of the culture supernatant was performed with Thin Layer Chromatography (TLC) after ethyl acetate extraction. Briefly, biomass of the isolates was removed from 7 days culture grown at different NaCl concentrations in CAS medium (7,500 × g for 15 min at 30°C), and supernatant was brought to pH 2.5 with HCl. This supernatant was then extracted with ethyl acetate, The extracted SA was evaporated to dryness in a vacuum evaporator, solubilized in a minimal vol of methanol and spotted on pre coated silica gel plates (Silica gel 60F 254; Merck). The chromatograms were developed in a solvent system consisting of chloroform: acetic acid: ethanol at the ratio of 95: 5: 2.5 (v/v). The plates were viewed in blue fluorescence emission under UV light (256 nm) immediately after removal from the developing chamber. The SA was detected by observing a UV reflected band with R_f value corresponding to that of the standard SA (Merck India).

To quantify SA production, the ethyl acetate extract was concentrated (1:3) under vacuum. SA concentration was determined by adding 5 μl of 2 M $FeCl_3$ and 3 ml of water to 1 ml of concentrated extract (Meyer and Hofte 1997). The absorbance of the purple iron-SA complex, which developed in the aqueous phase was measured at 527 nm and compared with standard curve of SA dissolved in ethyl acetate. The quantity of SA in the culture filtrate was expressed as mg ml^{-1}.

3.4.7 Zinc Solubilization

Isolates were screened for zinc solubilization ability on the basis of plate assay. 0.1% of insoluble zinc (ZnO or $ZnCO_3$) was amended in Tris minimal salt medium (supplemented with diverse NaCl concentrations). Isolates were spotted on the medium and incubated for 48 h. The degree of solubilzation by each isolates was determined by measuring zone of solubilzation (Fasim et al. 2002).

3.4.8 Exopolysaccharides Production

All the bacterial isolates were harvested by centrifugation for 10 min at 11,000 × g. The supernatant was filtered through 0.45-μm nitrocellulose membrane; two vol of cold ethanol were added to culture supernatants and stored overnight at 4°C. Precipitated material was collected by centrifugation (20 min at 2,500 × g) suspended in demineralized water, and mixed with 2 vol of cold ethanol. Samples were centrifuged (2,500 × g) and the pellets were dried at 100°C and weighed. The amount of EPS was expressed as polymer dry mass and expressed in g/l (Tewari and Arora 2014 a).

3.4.9 Hydrogen cyanide (HCN) Production

Production of HCN was observed according to Miller and Higgins (1970). The log phase culture of the isolates was spread on KB agar (supplemented with diverse NaCl concentrations) containing glycine (4.4 g/ l). Whatman No. 1 filter paper disc (9 cm in diameter) was soaked in 0.5% picric acid in 2% sodium carbonate (Na_2CO_3) and placed in upper lid of petri dish and incubated at 28ºC for 4 days. A change in color of filter paper from yellow to reddish brown was recorded as an index of cyanogenic activity.

3.4.10 Pyocyanin Production

The culture of the isolates in KB amended with salt concentrations (48 h) was centrifuged at 19319 × g and supernatant filtered through 0.22-μm pore. Absorbance of CFCS was measured spectrophotometrically at 690 nm and pyocyanin concentrations were calculated using the extinction coefficient for pyocyanin ($\varepsilon = 4{,}310\ M^{-1}\ cm^{-1}$ at pH 7) (Price-Whelan et al. 2006). The results were calculated as mean of three replicates

3.4.11 Enzymatic Activity

Protease secretion by the strains was judged on 1/10 King's B agar amended with 5 % (w/v) skimmed milk along with the varied concentrations of NaCl. The development of halo around inoculated area after 72 h of incubation at 28°C is indicative for exoprotease activity (Brownm and Fosterj 1970). The ability to produce cellulase was measured on plates containing minimal agar media with 2 % (w/v) 1-carboxymethylcellulose as carbon source (along with salt) (Hankin and Anagnostakis 1977) and observed for development of clear halo after 8 days of incubation of the colonies at 28° C. Extracellular chitinase activity was determined by spot inoculation on solid chitin minimal medium (CMM), whereas β- 1,3 glucanase enzyme was assayed according to Dunne et al. (1997) at varied salinity levels.

3.5 Phytopathogenic Fungal Strain

Phytopathogenic strain of *M. phaseolina* ARIFCC257 was procured from Mycology and Plant Pathology Group, Division of Plant Sciences, Agharkar Research Institute, Pune. The strain was grown and maintained on Potato dextrose agar (PDA) (Hi Media, Mumbai) at 28 and 4°C, respectively.

3.5.1 *In vitro* Biocontrol Activity Under Saline Conditions

Inhibitory activity of isolates against *M. phaseolina* was checked on DMM agar amended with different concentrations of NaCl (0–2,000 mM). Briefly, 6-mm mycelium disk of fungal pathogen was centrally placed on DMM plates, and 0.5 μl of exponentially grown wild and mutant strains were spot inoculated 1 cm away from the edge of the plate. The plates were incubated at 28°C for 6 days and percentage inhibition was determined (Arora et al. 2001).

The 48 h culture of the isolates in DMM broth amended with different concentrations of NaCl (0–2,000 mM) was centrifuged at 7,826×g for 15 min followed by membrane filtration (0.45 μm, Merck, India), and antifungal activity of cell free culture supernatant (CFCS) was detected by the well diffusion method (Gurusiddaiah et al. 1986). Briefly, spore suspension of *M.*

phaseolina in 0.85% saline was spread on DMM plates. A 6 mm well cut in DMM plates was filled with 50 µl of supernatant then incubated at 28°C for 48 h to observe zone of inhibition in millimeter (mm). Post interaction abnormalities in fungal mycelium were identified by taking fungal mycelium from the zone of inhibition and observing under phase contrast microscopy.

3.6 Molecular Characterization

On the basis of physiological stress studies two potent isolates PF23 and PF17 were selected from the collection and further identified on the basis of molecular characterization. DNA sequencing of isolate (PF23) was performed at Merk Biosciences Pvt Ltd, India. For sequence analysis of ~1.5 kb 16S rRNA gene fragment, consensus primers were used and amplification performed by Taq DNA Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. PCR mix composition contained:

Components	**Vol (µl)**
Genomic DNA	20 ng
dNTP mix (2.5Mm)	1.0 µl
Forward Primer	100 ng
Reverse Primer	100 ng
Taq buffer (10X)	1X
Taq Polymerase enzyme	3U
Glass distilled water	make up the vol 50µl

The sequence of the isolate was aligned by BLASTN search algorithm (Altschul et al. 1990) using combination of NCBI GenBank and Ribosomal Database Project (RDP) (Maidak et al. 1997; Maidak et al. 2000). The phylogenetic tree was constructed in MEGA 3.1 software using Neighbor Joining method (Saitou and Nei 1987).

3.7 Microbial Resource Center Accession Numbers

The sequence of the strain PF23 1.5 kb 16S rRNA gene sequence has been deposited in the NCBI GenBank Database under accession number KF598858.

3.8 PCR-ITS Analysis

For genomic DNA preparation chromosomal DNA from selected isolate PF17 showing fluorescence on *Pseudomonas* fluorescein agar was grown in KB broth at 28°C for 24 h. Cells were pelleted by centrifugation at 10,000 × g for 10 min. The pellet was washed and resuspended in 1ml TE buffer (10 mM Tris-HCl pH-8.0 and 1 mM EDTA pH-8.0) and centrifuged for 10 min at 10,000 × g, twice. The pellet was again resuspended in SET buffer (75 mM NaCl, 25 mM EDTA, pH 8.0 and 20 mM Tris-HCl pH-8.0) (500 µl). Lysozyme (10 mg/ml in 10 mM Tris-HCl pH-8.0) (10 µl) was added and cell suspension incubated at 37°C for 30-60 min. 0.3ºC of 10% sodium dodecyle sulphate (SDS) and 10 µl proteinase K (20 mg/ml n 50 mM Tris-HCl pH- 8.0) were then added to above suspension. After 60 min of incubation at 55°C, 0.3 vol of 5 M NaCl and equal vol of phenol, chloroform, isoamyl alcohol (25:24:1) were added, the suspension was incubated for 30 min at room temperature (RT). The aqueous layer was removed after centrifugation at 5000 rpm for 15 min. 0.1 vol of 3M sodium acetate and 1 vol chilled absolute ethanol was added to the aqueous solution and incubated at RT for 30 min followed by centrifugation at 10000 rpm for 10 min. The pellet was then washed by 70% ethanol and centrifuged at 10000 rpm for 10 min. This step was repeated two times. The pellet was dried then suspended in TE buffer and store at -40°C. To confirm strains as fluorescent *Pseudomonas*, 16S–23S rRNA intervening sequence specific primers ITS1F (AAGTCGTAACAAGGTAG); ITS2R (GACCATATATAACCCCAAG) were used to get an amplicon size of 560 bp. PCR reaction was carried out in 20 ml reaction containing 10 X buffer (with 2.5 mM $MgCl_2$), 2 ml; 2 mM dNTP mixture, 2 ml; 2 mM primer, 5 ml; Taq DNA polymerase, 3U; H_2O, 8 ml, and 50 ng of template DNA samples were amplified on DNA thermalcycler (DNA engine, MJ Research, USA) using the PCR conditions 92°C for 4 min, 28°C for 1 min and 72°C for 2 min. The total number of cycles was 40, with the final extension time of 10 min. The PCR products were resolved on 2% agarose at 50 V, stained with ethidium bromide (0.5 mg ml^{-1}) and photographed and analyzed using gel documentation system (Biorad, USA, model 2000, Quantity One software).

3.9 Pathogenicity Test

To check biosafety issues of PF23 and PF17 on human health, pathogenicity test was conducted on agar medium containing 2% sucrose, 0.5% yeast extract, 2% peptone, 2% KCl and 1.5% agar (Vermelho et al. 1996). The extra cellular proteases (caseinase, gelatinase and elastase) and hemolytic activities of the tested microorganisms on agar plates were checked, using different substrates; gelatin, casein, elastin and hemoglobin (1%). Agar medium individually amended with these substrates were poured (20 ml per plate) to harden. A loopful of the cultures was placed in the center of the agar plates. The plates were incubated at 37°C and observed for 10 days. The regions of enzymatic activity were detected as clear zone, indicating the hydrolysis of the substrate.

3.10 In *planta* PGP Activity and Suppression of Charcoal Rot of Sunflower

The study was conducted to detect PGP response and charcoal rot suppression ability of the strains taking sunflower as the crop under saline and non-saline conditions. Seeds of sunflower were surface sterilized for 2 min with 70% ethanol followed by 2% sodium hypochlorite (10 min). After thorough rinsing with sterile distilled water, the seeds were germinated in glass tubes of 50 ml capacity (2 seeds per tube), 1/3 filled with autoclaved plant growth media (PGM) (Engelke et al. 1987), supplemented with 125 mM NaCl (as sunflower seedlings displayed germination only up to 125 mM salinity level) and without NaCl in following sets of treatments: untreated seeds (control), seeds + *M. phaseolina* (negative control), seeds + PF17, seeds + PF23, seeds + PF17 + *M. phaseolina* and seeds + PF23 + *M. phaseolina.* Seeds were bacterized by dipping in cell suspension of respective strains (OD 610 = 0.1) for 10 min and dried overnight under aseptic conditions. *M. phaseolina* treatment was given by inoculating 10 µl of spore suspension in 0.85% saline on the surface of PGM after 2 days of seed application (Khare and Arora 2010). The experiment was conducted in five replicates and germination percentage was calculated after 5 days according to Ranal and Santana (2006). The extent of infection by *M. phaseolina* was indicated by the presence of dark brown lesions on root systems. Percentage disease incidence was calculated as percentage of diseased plants out of the total number of plants (Tewari and Arora 2014a).

3.11 *In vivo* PGP Activity and Suppression of Charcoal Rot of Sunflower

Experiment was conducted in small plastic pots (24 × 12× 12 cm) under controlled conditions (temperature 22 ± 2°C, humidity 70–80%, 12 h photoperiod) in plant growth chamber. Fungal inoculum was prepared on oat (*Avena sativa*) grains and added to the sterilized (autoclaved) sandy loam soil (pH 7.24) so as to get 10^4 fungal propagules/gm of soil before seed sowing (05 seeds/ pot). Sunflower seeds were dipped for 10 min in a cell suspension of isolate PF17 and PF23 (OD 610 = 0.1) mixed with carboxy methyl cellulose (CMC, 1%) and dried overnight in aseptic conditions. The experiment was conducted in sterilized soil in similar sets of treatments as *in planta* (tube) study, taking five replicates each. The effect of treatments was determined under control (received only normal irrigation water) and saline (irrigated as per requirement with 125 mM NaCl solution) conditions. Five plants from each set were taken randomly to determine root associated soil/root tissue ratio (RAS/RT). Plant watering was stopped 6 days before harvesting to facilitate the separation of root-associated soil from bulk soil. Other plant growth parameters (shoot and root length, fresh weight, dry weight, head diameter, and seed yield) and disease incidence were measured after 120 days.

3.12 PGP and Biocontrol Traits of PF23

3.12.1 Chemical Mutagenesis for Developing EPS-Defective Mutant

A loopful of PF23 cells were inoculated into 10 ml of DMM broth (Kragelund et al. 1997) and incubated at 28°C up to log phase. Subsequently, 100 μg ml^{-1} 5-bromouracil was added and further incubated for 02 h. Cells were centrifuged, washed thrice with sterile water, and resuspended in 10 ml of DMM broth, and mutants were fixed by overnight incubation at 28°C. The mutant bank was stored in 25% glycerol at - 40°C (Khare and Arora 2010). The clones of mutant bank were screened for EPS production according to Sandhya et al. (2009).

Mutagenic procedure worked effectively as EPS-defective mutant, $PF23^{EPS-}$ was repeatedly checked on DMM media for stability. The EPS defective mutants were further screened for salinity tolerance assay. The wild isolate PF23 served as a control in the mutant screening test. Maximum EPS-defective mutant was marked as $PF23^{EPS-}$.

3.12.2 Salinity Stress Assay

The log phase culture (OD 610 = 0.1) of PF23 and $PF23^{EPS-}$ were seeded in DMM broth, amended with different concentrations of NaCl (0 to 2, 200 mM) and incubated at 28°C. OD was measured at 610 nm by spectrophotometer up to stationary phase (120 rpm). The experiment was conducted in five replicates.

3.12.3 Effect of Saline Stress on EPS Production

To determine the effect of saline stress (0 to 2,000 mM) conditions on EPS content, selected strain PF23 and its EPS- defective mutant $PF23^{EPS-}$ were grown in DMM broth, with gradients of NaCl concentrations (0–2,000 mM) for seven days.

The amount of EPS was expressed as polymer dry mass and expressed in g/l (Fett et al. 1986; 1989). Total carbohydrate content was determined in the precipitated EPS according to Dubois et al. (1956) and the experiment was conducted in five replicates.

3.12.4 Composition of EPS

The precipitated EPS obtained from PF23 at different levels of salinity was hydrolyzed with 2 vol of 2.5 M H_2SO_4 at 100°C for 1 h. The solution was neutralized with 1 M sodium carbonate and spotted on the silica gel plate (Silica gel 60F 254; Merck).

The plate was developed in a TLC chamber using n-butanol: acetic acid: water (4:1:5 v/v) as the mobile phase at room temperature. The plate was dried, sprayed with alkaline potassium permanganate, and incubated at 100 °C for 10 min.

The Rf values of the colored spots were measured and compared with those of standard carbohydrates (glucose, mannose, fructose, mannitol, arabinose, xylose, rhamnose, raffinose, galactose, and trehalose) (Horborne 1976).

3.12.5 Fourier Transform Infrared Spectroscopy

The functional groups of EPS at different salinity (0 mM to 2000 mM NaCl) were recorded by Fourier Transform Infrared spectroscopy (FTIR) using Thermofisher scientific model Nicolet 6700 FTIR spectrophotometer. Sample was prepared by grinding with KBr pellets and it was scanned from 500 to 4000 cm^{-1}.

3.12.6 Scanning Electron Micrographs

Scanning electron microscopy (SEM) was done to study the surface morphology of EPS. EPS solution (1 mg/ml) was added to aluminium stubs and air dried. The sample was gold sputtered using SC7620 Sputtercoater device and analyzed by scanning electron microscopy (JEOL- JSM-6490 LV).

3.12.7 *In vitro* Antagonistic Activity of EPS

The antifungal activity of purified EPS obtained from PF23 (at varied salt concentrations ranging from 0 to 2,000 mM NaCl) was tested against *M. phaseolina* in separate flasks. For this, 500 µl spore suspension (OD 610 = 1.0) of *M. phaseolina* in 0.85% saline and 100 µg of EPS (obtained at different NaCl concentrations) was added in 50 ml of DMM broth, and incubated at 28°C for 7 days. In case of control, 500 µl of spore suspension was added in 50 ml of DMM broth. After 5 days, fungal mycelia were filtered (Whatman No. 1), oven dried at 70°C, and percentage inhibition was calculated.

Inhibition % = 1- [Dry weight of mycelium (sample)/ Dry weight of mycelium (control)] ×100.

Post interaction abnormalities in fungal mycelia were checked by taking treated fungal mycelium from the flask (treated with EPS) and were observing under phase contrast microscopy (Gupta et al. 1999; 2001).

3.12.8 SA production and extraction

Qualitative and quantitative analysis of SA production by $PF23^{EPS+}$ and $PF23^{EPS-}$ was done according to the method described by Meyer and Hofte (1997). Qualitative SA analysis of the culture supernatant was performed by TLC after ethyl acetate extraction. Briefly, biomass of both the bacterial strains were removed from 7 days old culture grown at different NaCl concentrations (0-600 mM NaCl) in CAS medium (7,500 ×g for 15 min at 30°C), and supernatant was brought to pH 2.5 with 0.1N HCl. The supernatant was then extracted with ethyl acetate in 1:1 ratio, extracted SA was evaporated to dryness in a vacuum evaporator, solubilized in a minimal vol of methanol and spotted on pre-coated silica gel plates (Silica gel 60F 254; Merck). The chromatograms were developed in a solvent system consisting of chloroform: acetic acid: ethanol in the ratio 95: 5: 2.5 (v/v). The plates were viewed in blue fluorescence emission under UV light (256 nm), immediately after removal from the developing chamber. The SA was detected by observing a UV reflected band with an R_f value corresponding to that of the standard SA (Merck India).

To quantify SA production, ethyl acetate extract was concentrated under vacuum. SA concentration was determined by adding 5 µl of 2 M $FeCl_3$ and 3 ml of water to 1 ml of concentrated extract (Meyer and Hofte 1997). The absorbance of the purple iron-SA complex, which developed in the aqueous phase, was measured at 527 nm and compared with a standard curve of SA dissolved in ethyl acetate. The quantity of SA in the culture filtrate was expressed as mg ml^{-1}.

3.12.9 High Performance Liquid Chromatography (HPLC)

For HPLC analysis 10 µl of extracted SA obtained from $PF23^{EPS+}$ and $PF23^{EPS-}$, at different salinity concentrations (0 to 600 mM NaCl) was injected into C18 column (25 × 4.6 mm) (Perkin Elmer, Model Flexar) and run under isocratic conditions using solvent system of 0.1% (v/v) orthophosphoric acid and 85% acetonitrile with flow rate 1 ml per min, wavelength set at 310 nm. Retention time (R_T) of the SA detected was compared with that of standard SA, and HPLC chromatograms were further compared with standard (Toiu et al. 2011).

3.12.10 Antagonistic activity of SA against *M. phaseolina*

The antifungal activity of purified SA obtained from $PF23^{EPS+}$ and $PF23^{EPS-}$ (at varied salt concentrations ranging from 0 to 600 mM NaCl) was tested against *M. phaseolina* in separate flasks. For this, 500 µl spore suspension (OD 610 = 1.0) of *M. phaseolina* in 0.85 % saline and 100 µg of SA (obtained at different NaCl concentrations) was added in 50 ml of CAS broth, and incubated at 28°C for 7 days. In case of control, 500 µl of spore suspension was added in 50 ml of CAS broth. After 5 days, fungal mycelia were filtered (Whatman No. 1), oven dried at 70°C, and percentage inhibition was calculated.

Inhibition % = 1- [Dry weight of mycelium (sample) / Dry weight of mycelium (control)] × 100

3.12.11 Post Interaction Abnormalities in Fungal Mycelium

Fungal mycelia were taken from the flask treated with SA and processed for SEM following the procedure of Weidenborner et al. (1989). Fungal mycelium after placing on cover glass were treated with 2 % osmium tetraoxide for 24 h at 20°C. The samples transferred to copper stubs over double adhesive tape were coated with gold in polaron, AU/PD sputter coater and scanned by SEM (JEOL-JSM-6490 LV) at 20 kV.

3.12.12 *In vivo* Pot Study

In vivo pot experiment was conducted in small plastic pots (24 × 12 × 12 cm) during the month of March-June for two consecutive years (2012 and 2013). Fungal inoculum was prepared on oat (*Avena sativa*) grains and added to the sterilized (autoclaved) sandy loam soil (pH 8.24) so as to get 10^4 fungal propagules/gm of soil before seed sowing (05 seeds/ pot). Sunflower seeds were surface sterilized by immersion in ethanol 95% for 30s, and in 5% NaClO for 10 min, followed by washing with sterile water. Sterilized seeds were dipped for 10 min in a cell suspension of isolate $PF23^{EPS+}$ and $PF23^{EPS-}$ (OD 610 = 0.1) mixed with 1% CMC, and dried overnight under aseptic conditions (Validov et al. 2007). Seed pelleting was done with purified SA and standard SA was done according to Mehran et al. (2013). The experiment was conducted in sterilized soil in following sets of treatments taking five replicates of each set: (i) non-bacterized seeds (control), (ii) $PF23^{EPS+}$, (iii) $PF23^{EPS-}$, (iv) *M. phaseolina*, (v) $PF23^{EPS+}$ + *M. phaseolina*, (vi) $PF23^{EPS-}$ + *M. phaseolina*, (vii) purified EPS, (viii) purified EPS + *M. phaseolina* , (ix) purified EPS + $PF23^{EPS+}$, (x) purified EPS + $PF23^{EPS+}$ + *M. phaseolina*, (xi) purified EPS + $PF23^{EPS-}$, (xii) purified EPS + $PF23^{EPS-}$ + *M. phaseolina*, (xiii) purified SA, (xiv) purified SA + *M. phaseolina*, (xv) purified SA + $PF23^{EPS+}$, (xvi) purified SA + $PF23^{EPS+}$ + *M. phaseolina*, (xvii) purified SA + $PF23^{EPS-}$, (xviii) purified SA + $PF23^{EPS-}$ + *M. phaseolina*, (xix) purified SA + purified EPS , (xx) purified SA + purified EPS + *M. phaseolina*, (xxi) standard SA, (xxii) standard SA + *M. phaseolina*. The effect of treatments was determined under control (receiving only normal water) and saline (irrigated as per requirement with 125 mM NaCl solution) conditions (Prıncipe et al.

2007). Other plant growth parameters including shoot length, root length, fresh weight, dry weight, head diameter and disease incidence were measured after 120 days of sowing.

3.13 Bioformulation Production

3.13.1 Selection of Carriers

For the preparation of bioformulation, locally available agricultural and industrial wastes were selected as carriers. Six solid carriers (corn husk, coriander husk, coconut husk, charcoal, saw dust and talc) were taken as supporting materials for monitoring the growth of isolates PF17 and PF23. All these carriers were radiation sterilized and left for overnight drying in air flow chamber. These carriers were also checked for physical parameters including, pH, inherent moisture content, water holding capacity according to Somasegaran and Hoben (1985).

3.13.2 pH Detection

Equal amounts of each solid carrier was mixed with distilled water and stirred thoroughly to form a slurry or paste and the pH was determined by digital pH meter (Page et al. 1982).

3.13.3 Inherent Moisture Content (IMC)

To analyze IMC, 10 g solid carrier was placed into an oven at 70°C for 24 h. The carrier material was weighed and kept again in the oven for 24 h to determine the end point of moisture loss. Moisture content was calculated by the following formula:

$$M = [(W_1 - W_2)/W_2] \times 100$$

Where, M = moisture content (%), W_1 = weight of carrier before drying, and W_2 = weight of carrier after drying (Aeron et al. 2011).

3.13.4 Water Holding Capacity (WHC)

WHC of the carrier was determined on dry weight basis. Water was added to 100 g of oven dried carrier material with continuous stirring until the carrier became saturated. The slurry was transferred in a measuring cylinder with a sieve (0.25 mm) covered drain hole at the bottom. The water was allowed to drain overnight from the carrier under normal conditions. After drying, the weight of the left-over carrier material in the cylinder was measured, and percent WHC was recorded according to Arora et al. (2008).

3.13.5 Tn5 Transposon Mutagenesis

Pseudomonas strain, $PF23^{strep+}$ indigenously resistant to streptomycin and $PF17^{amp+}$ spontaneous resistant to ampicillin were taken, for tracking the populations of bacteria in carriers; antibiotic resistance was also introduced in both the strains. *Escherichia coli* WA803 having suicidal plasmid (pGS9) integrated into a transposon Tn5 with a tetracycline-resistant and chloramphenicol - resistant marker gene was used to confer tetracycline resistance to $PF23^{strep+}$ and chloramphenicol resistance to $PF17^{amp+}$ respectively according to the method of Kumar et al. (2003).

3.13.6 Population Density in Different Carriers

Strain PF17 and PF23 were grown under, non-stress (0 mM NaCl) and stress conditions (200 mM NaCl). After growing osmotically stressed cells (SC) and unstressed cells (UC) (control) upto late exponential phase, cells were separated from supernatant by centrifugation and mixed in the respective carriers. Population of PF17 and PF23 in all the carriers were determined up to 06 months. The population density was measured by mixing 1 g of the carrier in 10 ml of distilled water aseptically and serially diluted to 10^{-6} and 10^{-7}. Average cell number per sample was calculated by estimating CFU on King's B agar amended with antibiotic.

3.14 Development of Diverse Formulation and Field Trials

3.14.1 Cell Based Bioformulation

The talc-based formulation from PF17 and PF23 were developed by following the method described by Vidhyasekaran and Muthamilan (1995). In brief, one kilogram of talc powder was taken and pH adjusted to neutral by adding $CaCO_3$ at the rate of 15 g/kg. The 400 ml of 7 days grown bacterial suspensions (stressed cells $_{(SC)}$ and unstressed cells $_{(UC)}$ of PF17 and PF23) were mixed separately with carrier-cellulose mixture under aseptic conditions. After drying (approximately 35% moisture content) overnight under sterile conditions. The prepared formulation from PF17 and PF23 were coated on surface sterilized seeds of sunflower.

3.14.2 Development of Cell Free Metabolite Based (CFMB) Formulation

The CFMB formulation from PF17 and PF23 were developed by separating cells form supernatant at 7836 × g for 10 min, under stressed and non-stressed conditions. CFCS of stressed and non-stressed cells were taken to design bioformulation. 400 ml of cell free culture supernatant (CFCS) was mixed separately with carrier-cellulose mixture under aseptic conditions. After drying (approximately 35% moisture content) overnight under sterile conditions. The prepared formulation from PF17 and PF23 were coated on surface sterilized seeds of sunflower.

3.14.3 Development of EPS Based Bioformulations

CFMB based EPS formulation was prepared by taking purified EPS from PF23 and talc powder amended with $CaCO_3$ in 1:1 ratio. Surface sterilized seeds of sunflower were soaked in suspension of talc-based EPS formulations, and then dried under shade (Arora et al. 2010a). Dextran was taken as a standard polysaccharide (reference) for coating the seeds.

3.14.4 Development of SA Based Bioformulation

For developing bioformulation, purified SA was taken and mixed with talc powder amended with $CaCO_3$ (1.5%) in 1:1 ratio. Designed formulation was coated on sunflower seeds, standard SA was taken as standard. Surface sterilized seeds of sunflower were soaked in suspension of talc-based SA formulations, and then dried under shade (Tavares et al. 2014).

3.14.5 Development of Cell + Metabolite Based Formulation

For developing cell plus metabolite based formulation purified EPS and purified SA were taken and mixed with PF23 cells. Metabolites and cells were mixed with talc powder amended with $CaCO_3$ (1.5%) in 1:1:1 ratio. Designed formulation was coated on sunflower seeds and dried under shade.

3.14.6 Development of metabolite (EPS + SA) based bioformulation

For the development of stress tolerating bioformulation combination of purified SA and purified EPS was taken and mixed with talc powder amended with $CaCO_3$ (1.5%) in 1:1:1 ratio. In the similar way combination of dextran and SA were taken in similar ratio and talc formulation was prepared.

3. 15 *In vivo* Field Trials

The field experiment was carried out in the semiarid regions of Kanpur Dehat (location 26° 20' 39.48" N and 79° 58' 1.85" E), soil having electrical conductivity 11 dS/m, pH 8.8), naturally infested with *M. phaseolina* (10^3 CFU/g soil) during the month of March to June for two consecutive years (2012 and 2013). The field trials were conducted using a randomized complete block design with five replicates of each treatment in a standard plot size of 100 m^2 (10 m × 10 m), where each block was 1.5 m × 1.5 m, with the developed bioformulations in following sets of treatment (pathogen infested salinized soil): (i) Control, (ii) $PF17_{(UC)}$, (iii) $PF17_{(SC)}$, (iv) $PF23_{(UC)}$, (v) $PF23_{(SC)}$, (vi) $PF17_{(UC)\ CFCS}$, (vii) $PF17_{(SC)\ CFCS}$, (viii) $PF23_{(UC)\ CFCS}$, (ix) $PF23_{(SC)\ CFCS}$, (x) $PF23_{(EPS)}$, (xi) Dextran, (xii) $PF23_{(SA)}$, (xiii) $Standard_{(SA)}$, (xiv) PF23 + EPS, (xv) PF23 + SA, (xvi) PF23 + SA, (xvii) Dextran + $Standard_{(SA),}$ (xviii) PF23 cells + EPS, (xix) PF23 cells + SA. The distance between each block was 0. 20 m and formulation coated seeds were sown in 30 cm row-width. The plots were irrigated as per requirement. Seed germination (%) was recorded on the 15th day after sowing (DAS). Vegetative growth parameters including biomass, root and shoot length, leaf area, chlorophyll content and seed yield were recorded 120 DAS (on harvest of crop). The data were subjected to analysis of variance, and means compared using Duncan's multiple range test (Gomez and Gomez 1984).

3. 16 Statistical Analysis

The data generated during quantitative evaluation of plant growth promotion values were analyzed by means of ANOVA, and means were compared by the Duncans Multiplicity Test Range (DMRT) using the SPSS software (ver. 10.1, SPSS Inc., www.spss.com). The significance level for all analysis was P = 0.05.

Chapter 4

Results

4.1 Bacterial Strains

110 fluorescent Pseudomonads were isolated from the rhizosphere of diverse plants. Three types of Pseudomonad colonies were observed. Eighty six isolates formed large fluorescent colonies with diffused margins and twenty isolates formed small, transparent, slightly fluorescent colonies (Figure 9). Remaining four isolates were intermediate in size and produced red pigment. Amongst all 24% isolates produced reddish - brown pigment on storage at 4ºC.

4.2 Phenotypic Characters

All the isolates were Gram negative and motile. Amongst all, forty three isolates were cocco-bacilli and sixty seven were rod shaped. Out of sixty seven rods, twenty one were slightly curved rods.

4.3 Biochemical and Physiological Characters

All the isolates were catalase and oxidase positive showed growth on 2 % NaCl and 8 % KNO_3. Out of all the isolates 59% produced acid from glucose . Gelatin hydrolysis was reported by 56% isolates while 89% produced fluorescein, and 90% formed fluorescent colony on *Pseudomonas* agar. Only 54% isolates were able to utilize citrate as carbon source (Table 2). The vernacular name 'fluorescent *Pseudomonas* group PF07-PF23' was coined for a group of 16 isolates obtained from the rhizosphere of sunflower showing characteristics that differentiated them from other members of fluorescent *Pseudomonas* species (Table 2).

4.3.1 Growth Kinetics

On the basis of growth study of isolates belonging to three different colony character groups, isolates with large fluorescent colonies had generation time 2.38 h. Pseudomonad strains producing brown pigment required 5.26 h for doubling of population. Strains forming small, less fluorescent colonies showed generation time of 4.01 h.

4.3.2 Biolog Analysis

Biolog analysis revealed that all the isolates of fluorescent group PF07-PF23 had similar carbon source utilization pattern (Table 3). PF23 showed 98% relatedness with *Pseudomonas aeruginosa* whereas, PF17 showed 97% similarity with *Pseudomonas fluorescence* in the carbon source utilization pattern of the defined database.

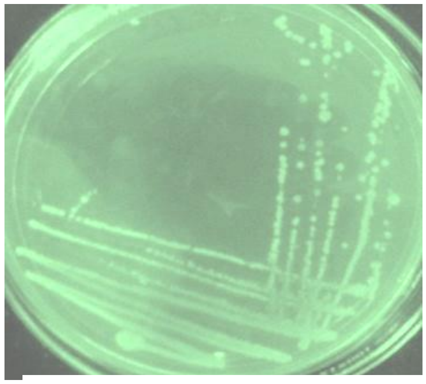
4.3.3 Intrinsic Antibiotic Resistance

The antibiotic sensitivity test of the isolates showed that all the isolates were sensitive towards the action of quinolone drugs. Isolates belonging to the group PF07-PF23 showed resistance towards penicillin and streptomycin derivatives, whereas, remaining were sensitive towards the action of both penicillin and streptomycin derivatives (Figure 10).

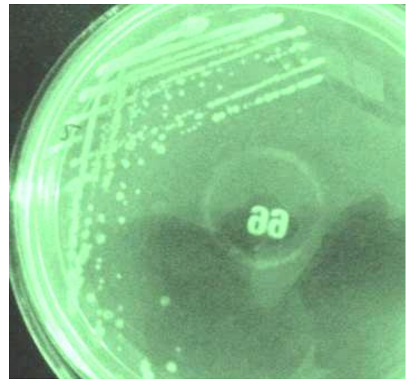
4.3.4 Stress Tolerance Assay

On the basis of stress tolerance assay it was found that PF23 and PF17 displayed maximum salinity, temperature and pH tolerance. Hence, these two strains were taken to perform further studies. PF23 showed upper limits of thermotolerance upto 45°C, osmotolerance upto 2000 mM NaCl (12%) and pronounced growth over wide range of pH (4-12).

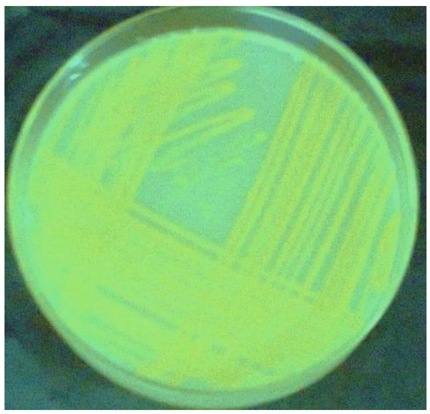
Salt shock with 100 mM concentration did not affect the growth of PF23, but higher osmotic stress of 500, 1000 and 1500 mM brought significant reduction in the OD by 32%, 48% and 68% respectively in comparison to non - stress (0 mM saline) conditions (Figure 11).



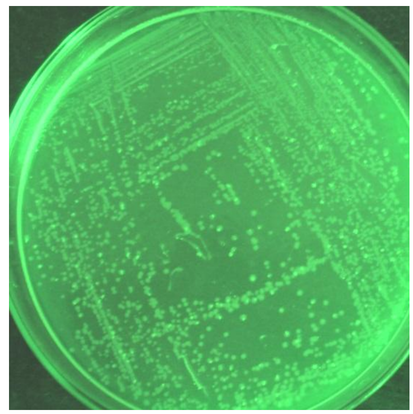
PF17



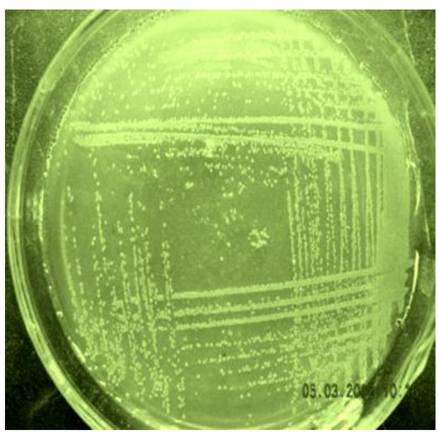
PF18



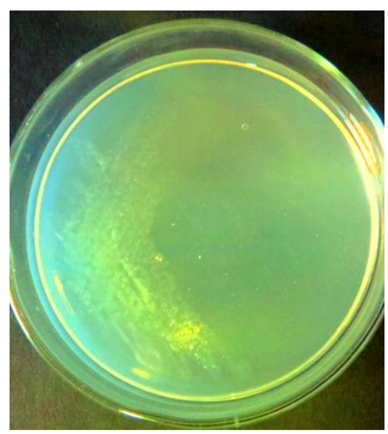
PF20



PF21



PF22



PF23

Figure 9: Fluorescent colonies of Pseudomonads on King's B agar medium

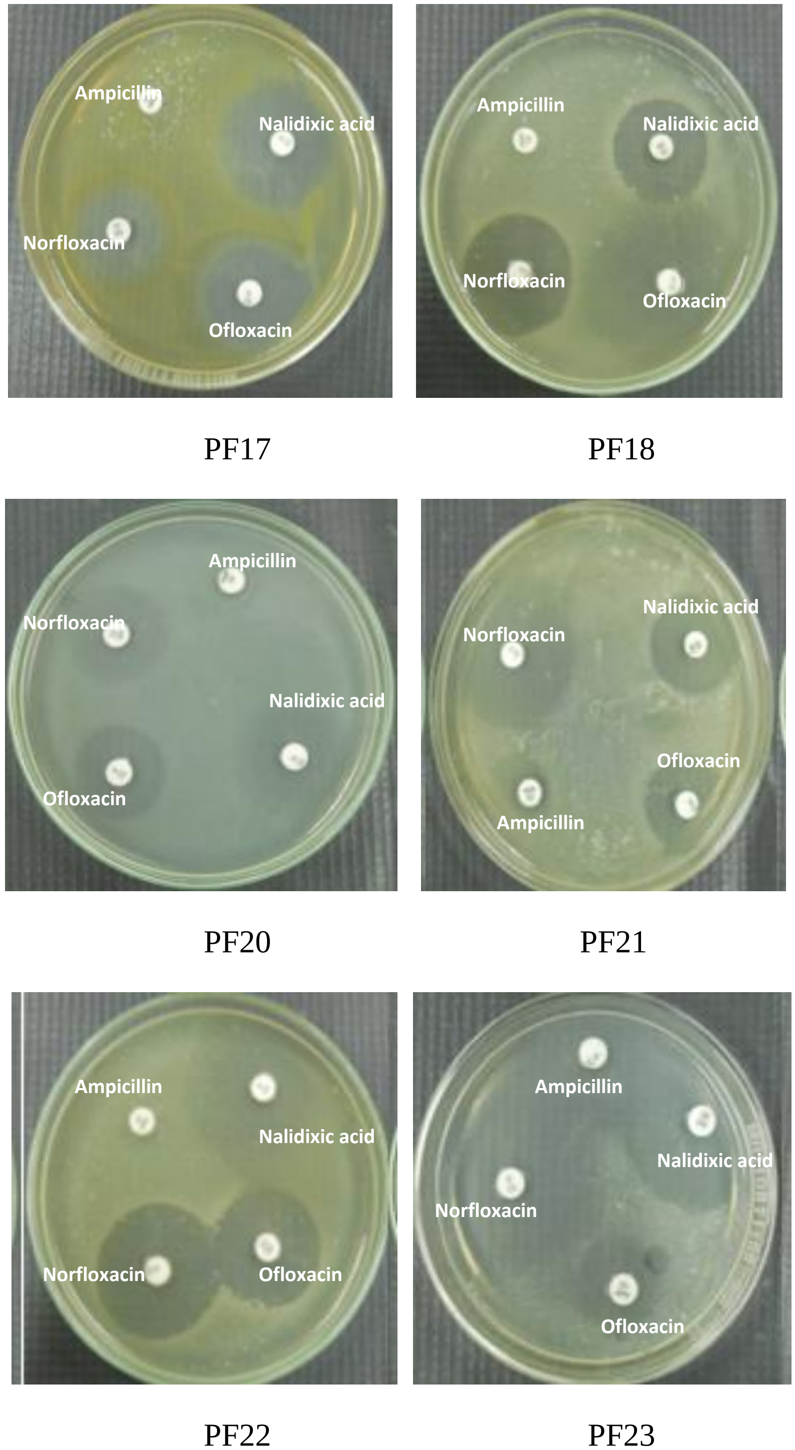


Figure 10: Intrinsic antibiotic resistance pattern of the isolates

Table 2: Biochemical Characterization of the Isolates

S. No.	Strains	Gram staining	Motility test	Catalase	Oxidase	Growth at 2% NaCl	Growth at 8% KNO_3	Growth at 4°C	Growth at 41°C	Acid from glucose	Gelatin hydrolysis	Starch hydrolysis	Citrate utilization	Nitrate reduction
1.	AMI	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
2.	AN2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
3.	AS1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
4.	AS2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
5.	AS3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
6.	BA1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
7.	BA2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
8.	BT1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
9.	BE1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
10.	BG1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
11.	BG2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
12.	CA1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
13.	CA2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
14.	CH1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
15.	CH2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
16.	CH3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
17.	CH4	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
18.	CO1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
19.	CO2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
20.	GA1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
21.	GA2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
22.	GA3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
23.	GI1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
24.	GN1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
25.	GN2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
26.	GN3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
27.	GN4	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve

28.	GU1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
29.	GU2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
30.	GL1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
31.	GL2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
32.	GL3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
33.	GR1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
34.	GR2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
35.	MA1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
36.	MA2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
37.	MA3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
38.	MA4	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
39.	MA5	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
40.	MR1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
41.	MR2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
42.	MR3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
43.	MR4	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
44.	AL1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
45.	AL2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
46.	AL3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
47.	AL4	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
48.	AL5	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
49.	AL6	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
50.	AL7	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
51.	AL8	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
52.	AL9	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
53.	MT1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
54.	MT2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
55.	MT3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
56.	MT4	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
57.	ML1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
58.	ML2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
59.	ML3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

60.	ML4	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
61.	ML5	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
62.	MU1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
63.	MU2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
64.	MU3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
65.	MU4	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
66.	MU5	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
67.	MU6	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
68.	NE1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
69.	NE2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
70.	PA1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
71.	PA2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
72.	PO1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
73.	PO2	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
74.	PO3	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
75.	PO4	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
76.	PO5	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
77.	PO6	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
78.	PO7	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
79.	PU1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
80.	RO1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
81.	SD1	-ve /coccobacillus	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
82.	**PF07**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
83.	**PF08**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
84.	**PF09**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
85.	**PF10**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
86.	**PF11**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
87.	**PF12**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
88.	**PF13**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
89.	**PF14**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
90.	**PF15**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
91.	**PF16**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**

92.	**PF17**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
93.	**PF18**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
94.	**PF19**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
95.	**PF20**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
96.	**PF21**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
97.	**PF22**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
98.	**PF23**	**-ve / rod**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**
99.	TA1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
100.	TA2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
101	TO1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
102	TO2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
103	TO3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
104	TO4	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
105	TL1	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
106	TL2	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
107	TL3	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
108	TL4	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
109	TL5	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve
110	TL6	-ve / rod	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve: isolates were negative for the test
+ve: isolates were positive for the test

Table 3: Carbon source utilization pattern of the Fluorescent *Pseudomonas* group PF07-PF23

C-Source	Results	C-Source	Results	C-Source	Results
α-Cyclodextrin	–	L-Rhamnose	–	D-Galactonic Acid Lactone	–
Dextrin	–	D-Sorbitol	–	D-Galacturonic Acid	–
Glycogen	–	Sucrose	–	D-Gluconic Acid	+
Tween 40	+	D-Trehalose	–	D-Glucosaminic Acid	–
Tween 80	–	Turanose	+	D-Glucuronic Acid	+
N-Acetyl-D-Galactosamine	–	Xylitol	–	α-Hydroxybutyric Acid	+
N-Acetyl-D-Glucosamine	+	Pyruvic Acid Methyl Ester	+	β-Hydroxybutyric Acid	+
Adonitol	–	Succnic Acid Mono-Methyl-Ester	+	γ-Hydroxybutyric Acid	–
L-Arabinose	+	Acetic Acid	+	p-Hydroxy Phenylacetic Acid	+
L-Arabitol	+	Cis-Aconitic Acid	+	Itaconic Acid	+
D-Cellobiose	+	D,L-Carnitine	+	α-Keto Butyric Acid	+
i-Erythritol	–	γ-Amino Butyric Acid	+	α-Keto Glutaric Acid	+
D-Fructose	+	Urocanic Acid	+	α-Keto Valeric Acid	+
L-Fucose	–	Inosine	+	Propionic Acid	–
D-Galactose	+	Uridine	–	Quinic Acid	+
Gentiobiose	+	Thymidine	–	D-Saccharic Acid	–

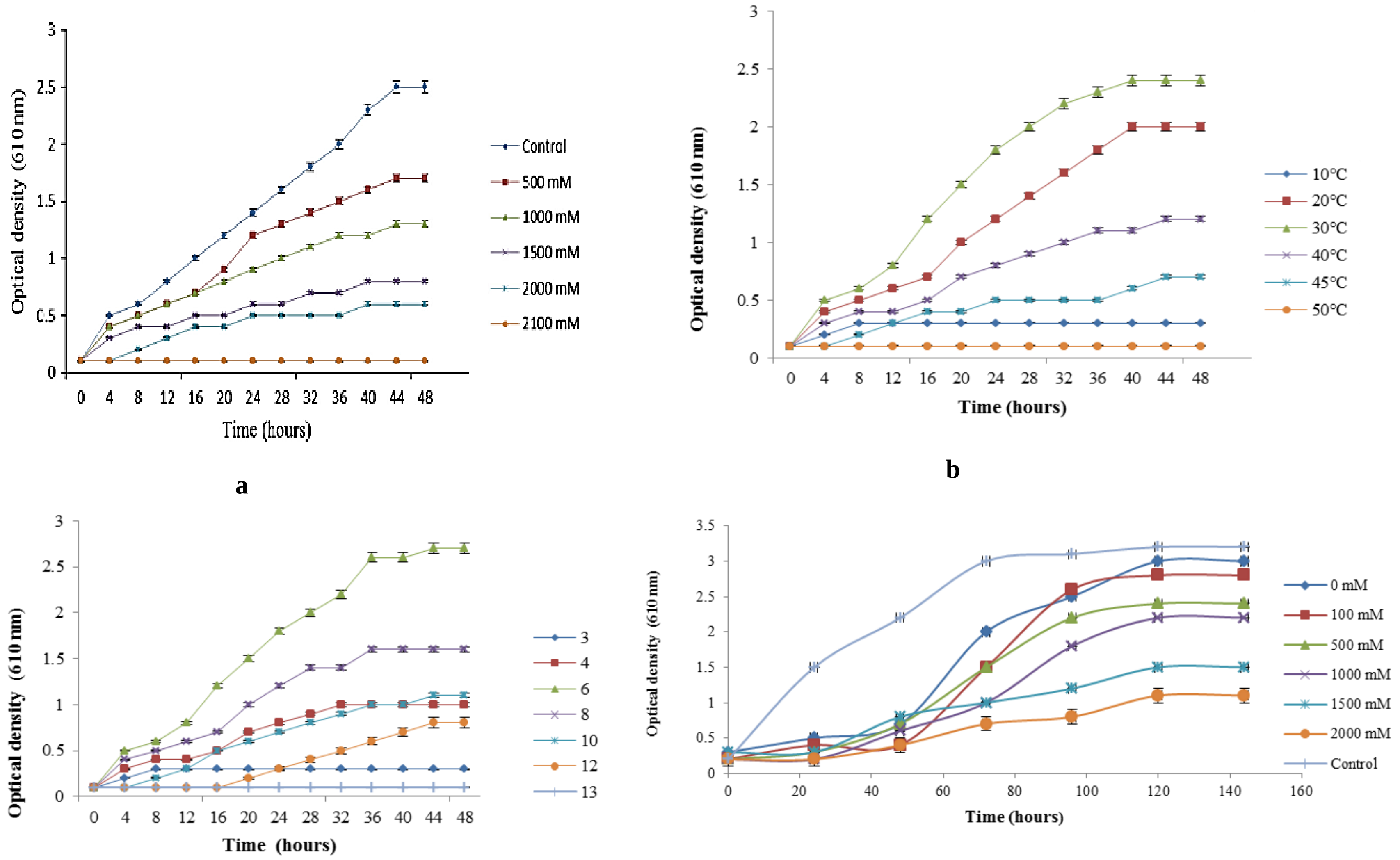
α-D-Glucose	+	Phenylethyl-amine	–	Sebacic Acid	+
m-Inositol	–	Putrescine	+	Succinic Acid	+
α-D-Lactose	–	2-Aminoethanol	+	Bromosuccinic Acid	+
Lactulose	–	2,3-Butanediol	–	Propionic Acid	–
Maltose	–	Glycerol	+	Succinamic Acid	+
D-Mannitol	+	Citric Acid	–	Glucuronamide	+
D-Mannose	+	Formic Acid	+	L-Alaninamide	+
D-Melibiose	–	D,L-α-Glycerol Phosphate	+	D-Alanine	–
β-Methyl-D-Glucoside	–	α-D-Glucose-1-Phosphate	–	L-Alanine	+
D-Psicose	–	D-Glucose-6-Phosphate	+	L-Alanyl-glycine	+
D-Raffinose	–	Glycyl-L-Glutamic Acid	–	L-Asparagine	+
L-Aspartic Acid	+	L-Histidine	–	L-Leucine	+
L-Glutamic Acid	–	Hydroxy-L-Proline	+	L-Ornithine	–
Glycyl-L-Aspartic Acid	–	L-Pyroglutamic Acid	–	L-Phenylalanine	–
L-Proline	+	D-Serine	+	L-Serine	_
L-Threonine	_	D, L-Lactic acid	–		

-ve: isolates were negative for the test
+ve: isolates were positive for the test

PF23 did not show growth above 2,000 mM salt concentration. Another feature that has to be outlined is that the strain showed thermotolerance upto 45°C. While raising the temperature from 20°C to 30°C there was 20% increment in OD of bacterial cells, while further increase in temperature to 40°C and 45°C brought 50% and 71% reduction in OD, whereas there was 96% reduction in growth at 50°C (Figure 11 b-c). PF23 displayed growth over a wide range of acidic, alkaline and neutral pH (ranging from 4-12). PF23 survived best at pH 7 and there was 66% and 73% reduction in OD at pH 4 and pH 12.

On the other hand isolate PF17 displayed salt tolerance upto 1400 mM NaCl. NaCl concentration upto 200 mM NaCl didn't affect the growth pattern of PF17 but further increase in salinity brought reduction in growth. No growth was observed beyond 1400 mM NaCl. Thermotolerance capacity of the isolates was recorded upto 50°C and pH tolerance ranged from 3-13 (Figure 12 a-c).

Isolate PF23, maintained its survivability when multiple stresses were given simultaneously (combined stress of 2000 mM NaCl at pH 12.0 incubated at 45°C) (Figure 12d). Results show that when multistress conditions were provided simultaneously, initially bacterial cells acclimatized and then growth picked up after 48 h. However, PF17 displayed initial acclimatization after 72h in multistress growth assay followed by further increase in growth (Figure 12d).



a b

C d

Figure 11: Effect of stress conditions on growth of PF23

a: salinity stress, b: temperature stress, c: pH stress;

d: multistress (PF23 with different NaCl Concentrations adjusted with pH 12 and incubated at 45°C

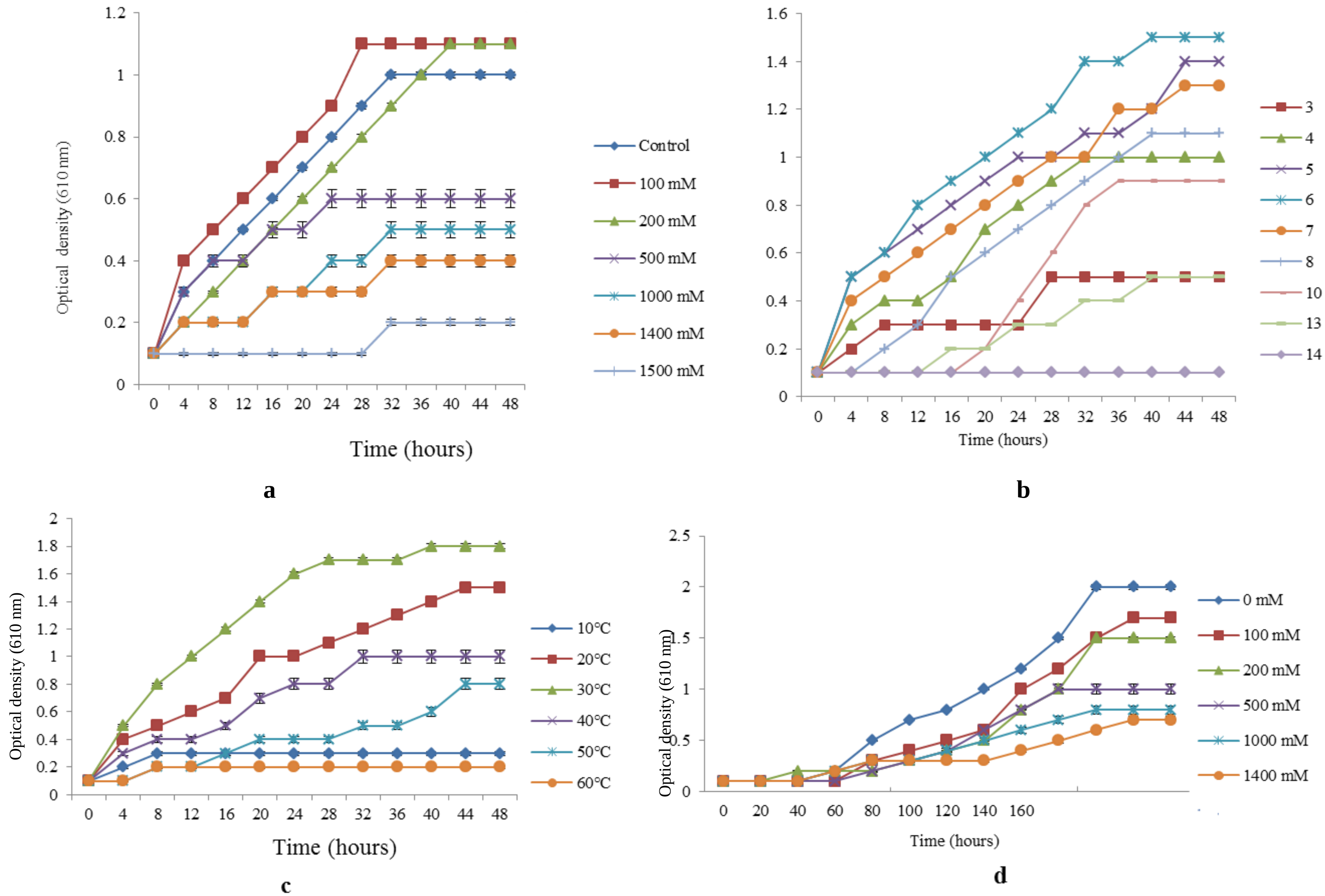


Figure 12: Effect of stress conditions on growth of PF17

a: salinity stress, b: temperature stress, c: pH stress;

d: multistress conditions PF17 with different NaCl Concentrations adjusted with pH 13 and incubated at 50°C

4.4 Plant Growth Promoting and Biocontrol Traits

4.4.1 Phosphate Solubilization

PF17 showed phosphate solubilization ability on PKV agar medium as indicated by development of clear zone upto 600 mM salinity level. Under control conditions (0 mM) SI was recorded to be 1.29 that was maintained significantly similar upto 200 mM NaCl, further increase in salinity at 300, 400, 500 and 600 mM displayed SI of about 1.12, 1.05, 0.7 and 0.3 respectively (Figure 13 a). The results of quantitative examination also showed similarity with qualitative analysis. Under non-saline conditions PF17 solubilized 576 µg/ml phosphate that was significantly similar upto 200 mM NaCl, further increase in salinity brought steep reduction in solubilzation (Figure 14). No solubilization was observed above 600 mM NaCl concentration. However, quantitative and qualitative analysis showed that PF23 was unable to solubilize phosphate under control (non -saline) and saline conditions.

4.4.2 Indole-3-Acetic Acid

Pseudomonas isolate PF23 displayed IAA production of 1.9 µg/ml under control conditions however, further increase in salinity completely inhibited IAA production. Isolate PF17 showed IAA production upto 600 mM NaCl. Under non - saline conditions (0 mM salinity) IAA by PF17 was recorded to be 6.8 µg/ml. This concentration was maintained as such upto 200 mM NaCl, whereas further increase in salinization resulted in steep decline in IAA production (Figure 13 d and 14).

4.4.3 Siderophore

PF17 and PF23 displayed siderophore production on CAS agar as indicated by development of orange halo surrounding the colony under control conditions (Figure 13b). Quantitative analysis showed that hydroxymate type siderophore was produced by PF17 and PF23 at a concentration of 127.5 μM and 48 μM respectively. Both the isolates failed to show catecholate siderophore production under saline or non-saline conditions.

Both qualitative and quantitative analysis showed that isolate PF23 displayed siderophore production only under control (0 mM NaCl) conditions, whereas, PF17 displayed siderophore upto 600 mM NaCl. PF17 displayed siderophore production significantly similar to that under control state (0 mM NaCl) upto 200 mM NaCl (Figure 14).

4.4 Salicylic Acid Production

Qualitative and quantitative analysis suggested that PF23 displayed SA production upto 500 mM NaCl concentration. The SA produced by the PF23 was confirmed by the blue bands that appeared on pre-coated silica gel, when viewed under UV illumination. A fluorescent blue spot in the sample co-migrated with standard of SA, which also displayed same fluorescence. The R_f value of SA (0.61) produced by the PF23 at different NaCl concentrations matched with the R_f value (0.60) of the standard SA.

Thickness of the SA band obtained at 0 and 100 mM NaCl showed exact resemblance with authentic SA, however, further increase in salinity brought fader and thinner bands up to 500 mM salinity. No SA production was observed beyond 500 mM by PF23.

Quantitative analysis further authenticated qualitative results. Under control conditions (at 0 mM NaCl) PF23 produced 6.14 mg ml^{-1} of SA, which was significantly similar to the SA obtained at 100 mM salinity (6.12 mg). However, there was 16.2%, 70.5% and 90% reduction in SA production observed on increasing salinity level to 200 mM, 300 mM and 400 mM NaCl respectively. However, no SA production was shown by PF17 under non-saline or saline conditions.

4.4.5 Zinc Solubilzation

PF17 showed clear zone in plate assay upto 600 mM NaCl thereby confirming the role of zinc solubilzation by the isolate. Measured zone of solubilzation was recorded to be 9 mm, 7 mm, 5 mm, 3 mm and 2 mm at 0 mM, 300 mM, 400 mM, 500 mM and 600 mM salinity respectively. Solubilzation upto 200 mM salinity was maintained similar as obtained at 0 mM. However, no zinc solubilization activity under saline or non-saline conditions was shown by PF23 (Figure 13).

4.4.6 Exopolysaccharide Production

PF23 displayed high EPS production with progressive increase in salinity (Figure 15). At 0 mM NaCl conditions there was minimum EPS produced by the PF23 that become apparently high with progressive increase in salinity upto 2000 mM NaCl conditions.

There was an increase in EPS production by about 26, 38 and 66% at salinity level of 1,000, 1,500, and 2,000, respectively, in comparison to control (Table 6). No EPS was recorded under non- saline and saline conditions by PF17.

4.7 Hydrogen Cyanide Production

Isolate PF17 showed change in color of filter paper to brown upto 600 mM NaCl concentration and was positive for HCN production (Figure 13c). Whereas, PF23 was negative for HCN production both under saline or non - saline state.

4.4.8 Pyocyanin Production

PF17 displayed pyocyanin production upto 600 mM NaCl. Under control conditions PF17 produced 28 µM of pyocyanin that was maintained significantly similar upto 200 mM NaCl. Further increase in salinity, brought steep reduction in pyocyanin. However, no pyocyanin was produced by PF23 under non- saline or saline conditions (Figure 14).

4.4.9 Enzymatic Activity

Lipase activity was detected in the form of opacity around the colony of the isolate PF17 due to the formation of insoluble calcium soaps. This enzymatic activity was maintained upto 600 mM salinity level in PF17. PF17 also displayed chitinase and β -1, 3 glucanase activity upto 600 mM NaCl. Whereas no lipase, chitinase and β -1, 3 glucanase activity was observed by PF23 under saline or non - saline conditions. Both the isolates were negative for cellulase and exoprotease production under saline or non - saline conditions.

4.5 *In vitro* Biocontrol Activity Under Saline Conditions

Strain PF23 and its CFCS demonstrated 90 and 88% inhibition of *M. phaseolina* at 0 mm NaCl concentration, respectively. Upto 100 mM NaCl the percentage of inhibition was maintained similar to that at 0 mM NaCl. Biocontrol ability of PF23 was maintained up to 500 mM. Cells and CFCS of PF23 showed 54 and 51% inhibition of *M. phaseolina* at 500 mM NaCl. Further increase in salinity ceased inhibitory activity of PF23 (Figure 16a).

On the other side PF17 and its CFCS displayed inhibitory spectrum against *M. phaseolina* upto 600 mM NaCl concentration. At 0 mM NaCl cells and CFCS of PF17 displayed 75% and 71% inhibition of phytopathogen which was maintained significantly similar upto 200 mM salinity. Further increase in salinity to 300, 400, 500 and 600 mM NaCl brought 60%, 40%, 30% and 20% reduction in the growth of phytopathogen (Figure 16b).

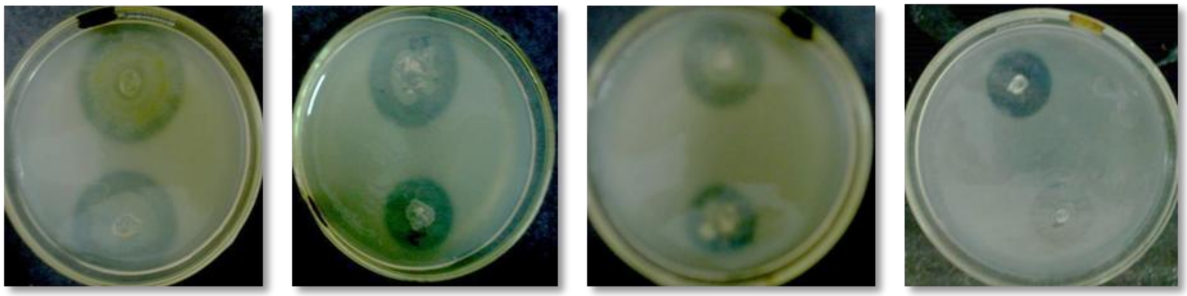
Post interaction abnormalities in fungal mycelium were observed under phase contrast microscope. It was found that PF17 and PF23 caused halo cell formation, mycelial deformities and hyphal tip degradation of *M. phaseolina.* Digestion of fungal cell wall along will shriveling and curling of mycelium is also clear from the photographs (Figure 17). Sclerotial development in *M. phaseolina* was also arrested.

4.6 Molecular Characterization

The comparison of complete 16S rRNA gene sequence (Figure 18a) of the isolate PF23 with the sequence of the other strains of the genus *Pseudomonas* showed 99 % relatedness to *P. aeruginosa* (Gen Bank accession number AB691548.1 and KC417305.1) (Figure 18b and 18c). Based on biochemical, physiological characteristics, and nucleotide homology, isolate PF23 was identified as *Pseudomonas aeruginosa* and sequence data submitted to NCBI Gen Bank with accession number KF598858.

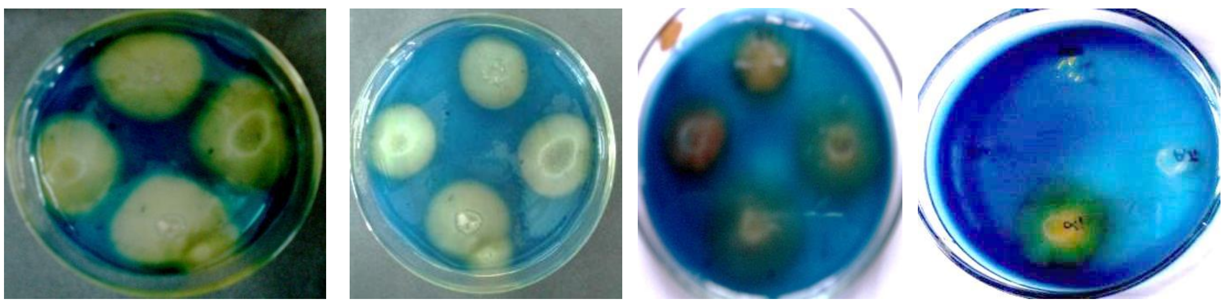
4.7 PCR-ITS Analysis

The 16S–23S ITS region from PF17 isolates gave a single amplicon of size of 560 bp, which confirmed that the isolate was *Pseudomonas fluorescens* (Figure 19).



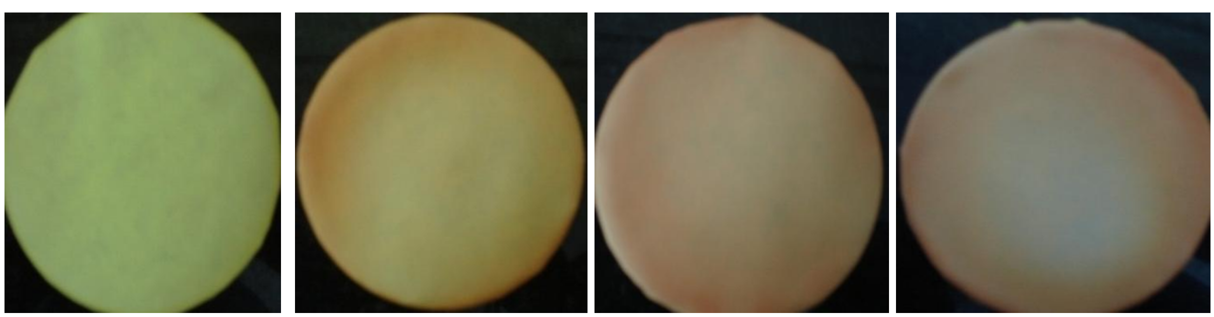
(a) 0 mM NaCl (b) 200 mM NaCl (c) 400 mM NaCl (d) 600 mM NaCl

A: Phosphate solubilization



(a) 0 mM NaCl (b) 200 mM NaCl (c) 400 mM NaCl (d) 600 mM NaCl

B: Siderophore production

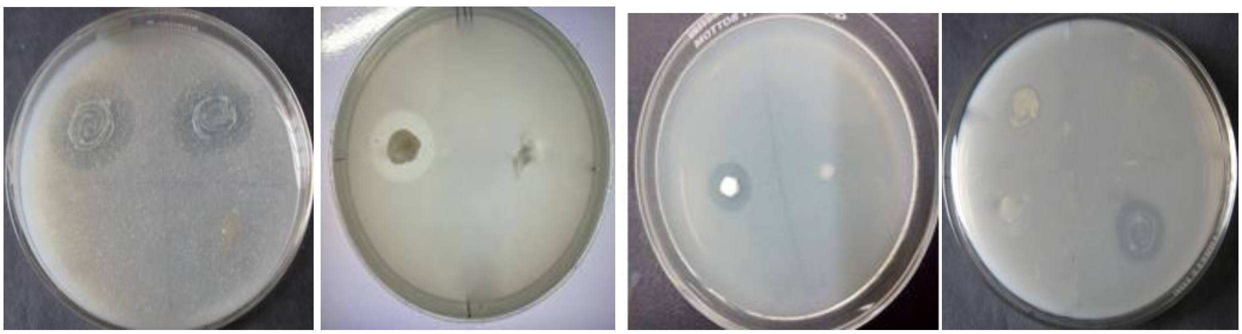


(a) 0 mM NaCl (b) 200 mM NaCl (c) 400 mM NaCl (d) 600 mM NaCl

C: HCN production

a) 0 mM NaCl (b) 200 mM NaCl (c) 400 mM NaCl (d) 600 mM NaCl

D: IAA production



a) 0 mM NaCl (b) 200 mM NaCl (c) 400 mM NaCl (d) 600 mM NaCl

E: Zinc solubilzation

Figure 13: PGP and biocontrol traits displayed by PF17 under saline conditions

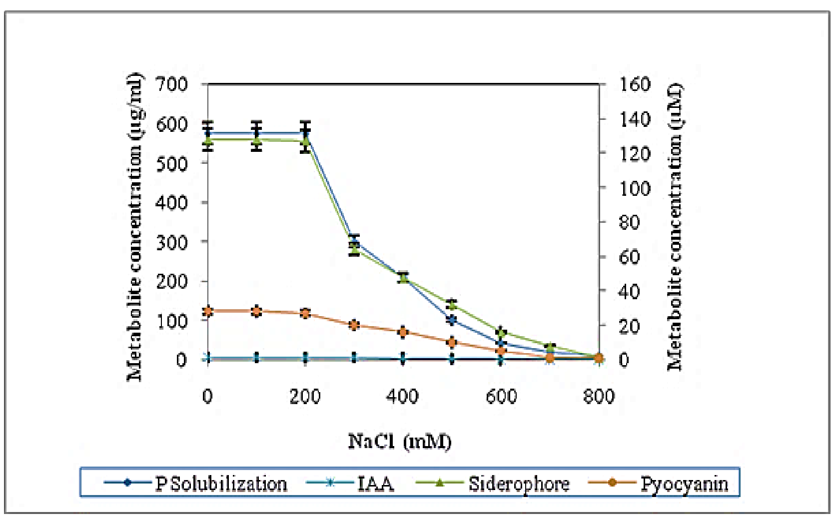


Figure 14: PGP and biocontrol metabolites produced by PF17 under saline conditions

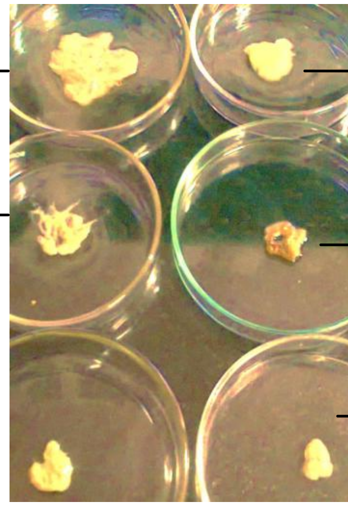
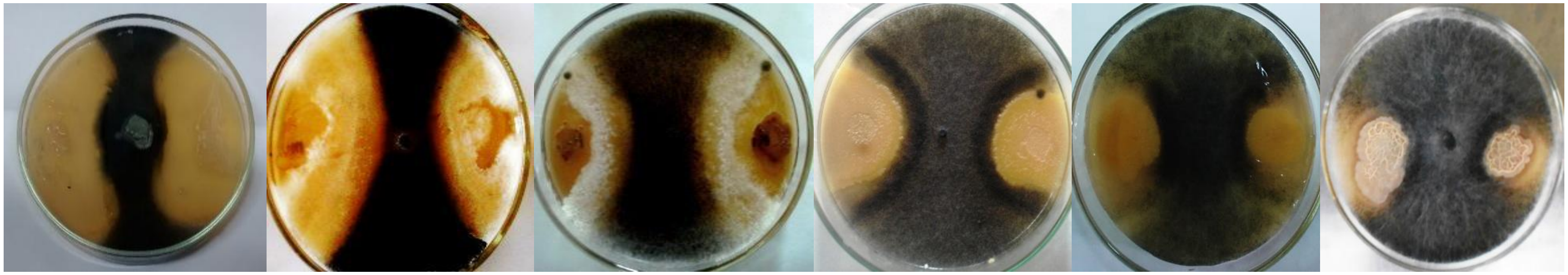
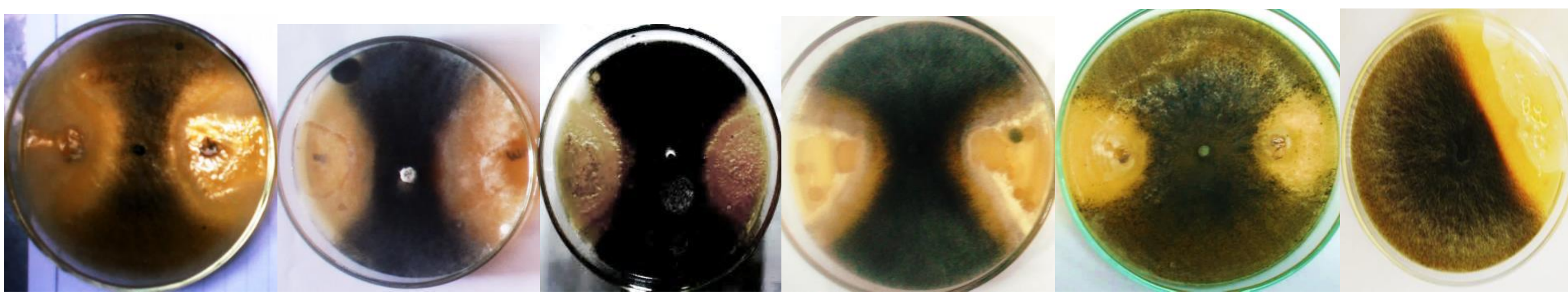


Figure 15: EPS production by PF23 under saline conditions



0 mM 100 mM 200 mM 300 mM 400 Mm 500 mM

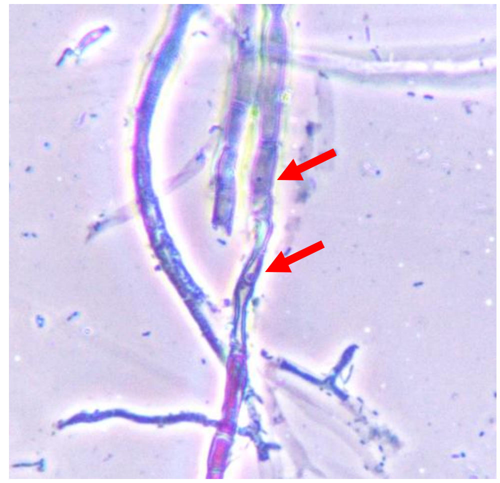
Figure 16 a : Biocontrol ability of PF23 against *M. phaseolina* under saline conditions



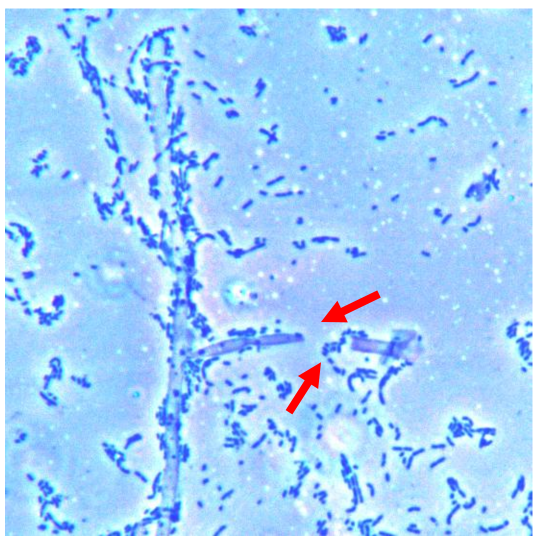
0 mM 200 mM 300 mM 400 mM 500 Mm 600 mM

Figure 16 b: Biocontrol ability of PF17 against *M. phaseolina* under saline conditions

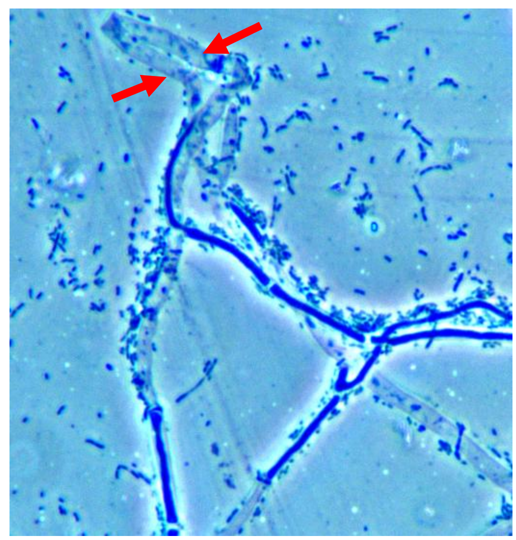
A



B



C



D

A: Halo formation, B: deformity in fungal mycelium,
C: digestion of fungal cell wall, D shriveling and curling of mycelium

Figure 17: Post interaction morphological changes in *M. phaseolina* due to PF17

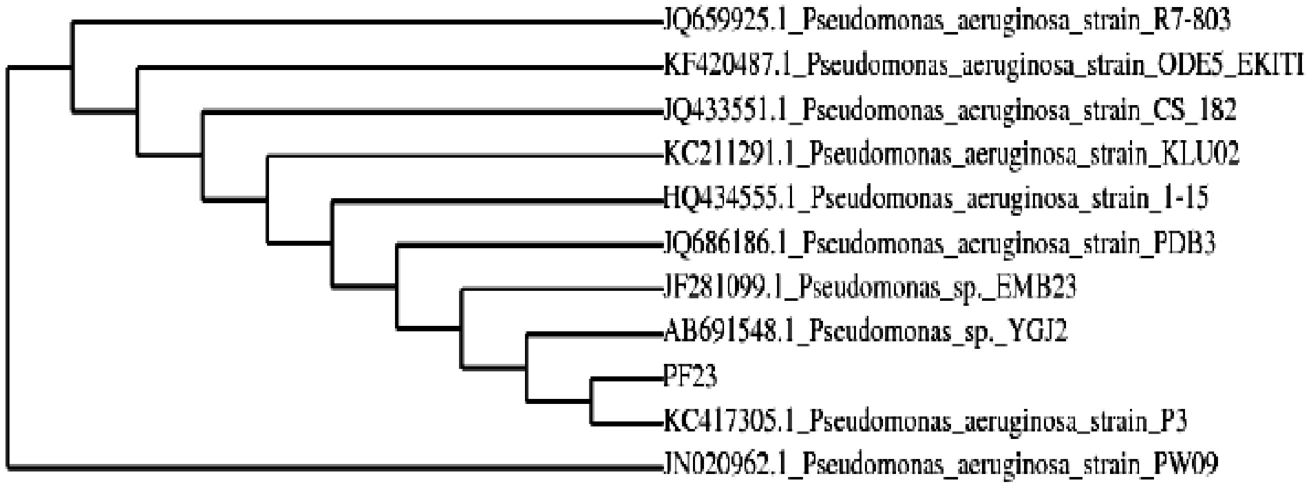


Figure 18a: Phylogenetic tree using neighbor joining method

Aligned Sequence Data of: (1403 bp)

TGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAG
TGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTA
TCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGAT
GATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC
CTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAA
TACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGG
AACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAG
GAAGGAACACCAGTGGCGAAAGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGAT
AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGG
GAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCC
TTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAG
CTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAA
TCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAG
CTAGTCTAACCGCAAGGGGGACGGTT

Figure 18b: Aligned sequence data of 1403 bp

Alignment View	ID	Alignment results	Sequence Description
	PF23	1	Studied sample
	KF420487.1	0.99	*Pseudomonas aeruginosa strain ODE5_EKITI*
	JQ433551.1	0.99	*Pseudomonas aeruginosa strain CS_182*
	KC417305.1	0.99	*Pseudomonas aeruginosa strain P3*
	KC211291.1	0.99	*Pseudomonas aeruginosa strain KLU02*
	JQ686186.1	0.99	*Pseudomonas aeruginosa strain PDB3*
	JQ659925.1	0.99	*Pseudomonas aeruginosa strain R7-803*
	AB691548.1	0.99	*Pseudomonas sp. YGJ2*
	JN020962.1	0.50	*Pseudomonas aeruginosa strain PW09*
	HQ434555.1	0.99	*Pseudomonas aeruginosa strain 1-15*
	JF281099.1	0.99	*Pseudomonas sp. EMB23*

Figure 18c : Alignment view using combination of NCBI Genebank and RDP database

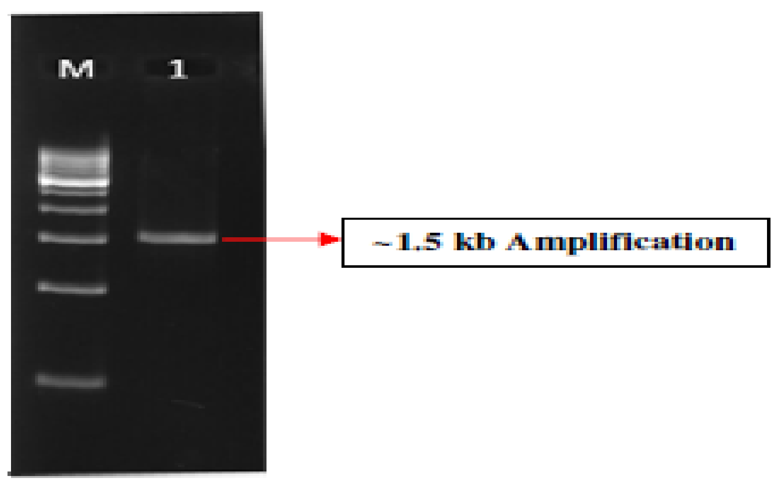


Figure 19: ITS-PCR region

4.8 Pathogenecity Test

Both the isolates PF23 and PF17 did not display protease, gelatinase and elastase activity as no zone of clearance was observed around the colony after 10 days of incubation. Strains didn't show hemolytic activity on hemoglobin supplimented medium, therby suggesting that the strains were non –pathogenic and avirulent.

However, standard strain of *P. aeruginosa* ATCC19429 taken for comparision displayed clear zone around the colony when supplemented with casein, gelatin, elastin and hemoglobin suggesting it to be a potent pathogen. In vivo lab experiments have proved the avirulent nature of PF23 and PF17 whereas, clinical trials will be done soon .

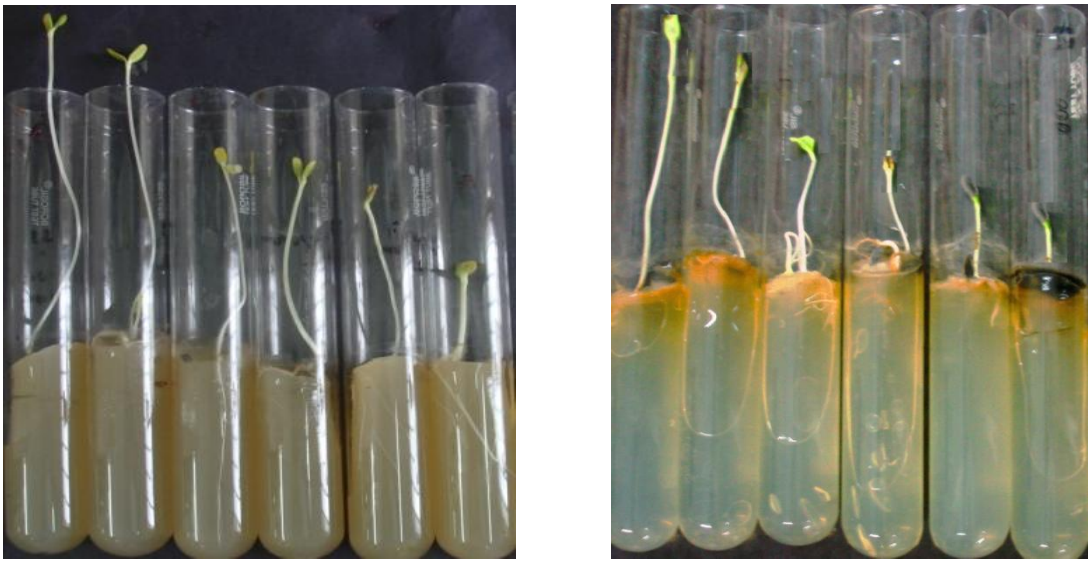
4.9 *In planta* PGP Activity and Suppression of Charcoal Rot of Sunflower

Seed biopriming with PF23 and PF17 showed significant increase in germination in comparison with non-primed seeds (control) both under saline (125 mM NaCl) and non-saline (0 mM) conditions. Seed biopriming with PF23 brought 25 and 50% increment in germination in comparison with unprimed seeds under non-saline and saline conditions, respectively. Treatment with PF23 brought increment in root length, shoot length, fresh weight, dry weight by 105, 59, 115, 212%, respectively, in comparison with control (untreated seeds) under saline stress (Figure 20). Under non-saline conditions it was observed that PF23 brought 100, 31, 46, and 117% enhancement in root length, shoot length, fresh weight, and dry weight, respectively (Table 4). In presence of *M. phaseolina*, PF23 brought increment in fresh weight and dry weight by 103 and 84% in comparison with unbacterized seeds, under saline conditions. In comparison with non-bacterized control, PF23 showed 80 and 70% reduction of disease incidence in non-saline and saline conditions respectively (Table 4).

PF17 also displayed increase in plant growth parameters of sunflower in both the absence and presence of pathogen, under saline and non-saline conditions. Seed treatment with PF17 brought enhancement in dry weight by 103.2% and 194.4% under non-saline and saline conditions in comparison to control. PF17 even showed 74.3% and 67.1% reduction of disease incidence in non-saline and saline conditions respectively.

4.10 *In vivo* PGP Activity and Suppression of Charcoal Rot of Sunflower

Results of *in vivo* study showed that treatment of seeds with PF17 and PF23 caused significant increase in plant growth parameters including root length, shoot length, dry weight, RAS/RT and head diameter under saline and non-saline conditions over untreated seeds in the presence as well as in the absence of phytopathogen (Table 5 and Figure 21). Biopriming of seeds with PF17 and PF23 brought 34.4% and 50% increase in seed yield under saline conditions, in comparison with unbacterized seeds. *M. phaseolina* caused 79 and 81% incidence of disease in non-saline and saline conditions (Figure 21b). PF23 resulted in 71 and 63% reduction of disease incidence in comparison with non-bacterized control under non-saline and saline conditions, respectively. Whereas, PF17 caused 59.4% and 51.9% suppression of disease incidence under non-saline and saline conditions in comparison to control. In the presence of pathogen, PF17 and PF23 brought 109.4% and 138.0% enhancement in seed yield under saline conditions, respectively, in comparison with *M. phaseolina*-infested untreated seeds.



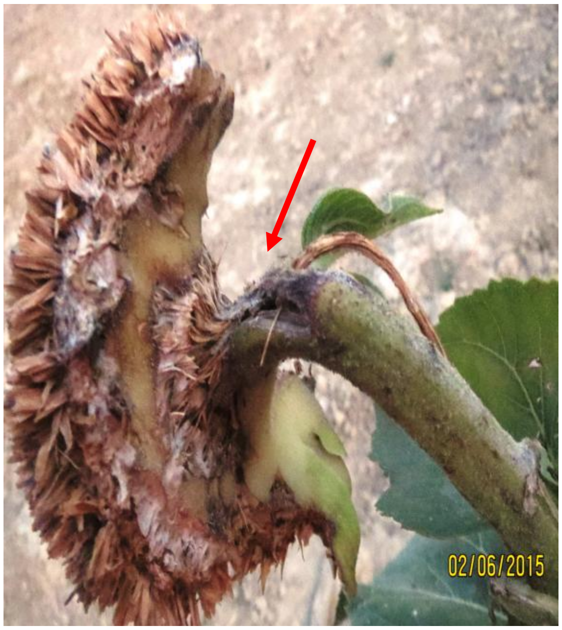
a b c d e f a b c d e f

Non – saline conditions (0 mM NaCl) Saline conditions (125 mM NaCl)

a: PF23, b: PF17, c:PF23 + *M. phaseolina*, d: PF17 + *M. phaseolina*,
e: Untreated seeds (positive control), f: *M. phaseolina* (negative control)

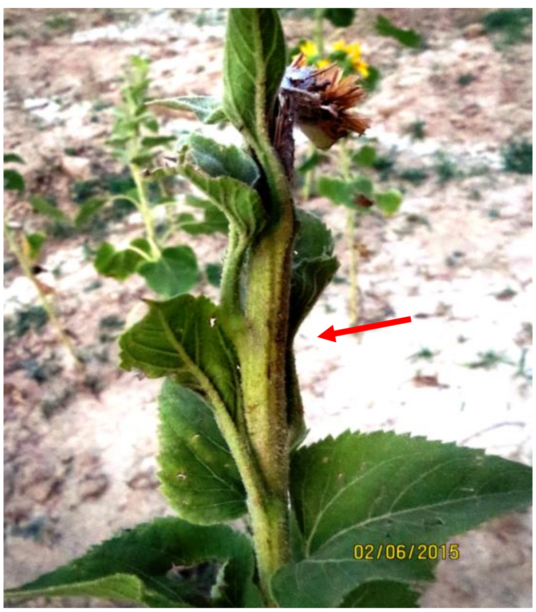
Figure 20: *In planta* PGP activity and suppression of charcoal rot of sunflower

Figure 21 a: *In vivo* pot study under saline conditions



Head Rot

Leaf rot



Stem rot

Leaf discoloration

Figure 21b: Disease incidence in sunflower due to *M. phaseolina* (in pot study)

Table 4: Influence of *Pseudomonas* treatments on growth of sunflower plants in both absence and the presence of *M. phaseolina*, under non-saline (control) and saline (*in planta*) conditions

	Non - saline conditions (0 mM NaCl)					**Saline-conditions (125 mM NaCl)**				
Treatments	**Germination %**	**Root length**	**Shoot length**	**Fresh weight**	**Dry weight**	**Germination %**	**Root length**	**Shoot length**	**Fresh weight**	**Dry weight**
Unbacterized seeds	80±0.01^{b}	3.0 ±0.02^{b}	11.5 ±0.01^{b}	1.44±0.02^{b}	0.249 ±0.03^{b}	60 ±0.02^{b}	2.0±0.01^{b}	7.6±0.02^{b}	0.75±0.02^{b}	0.126 ±0.01^{b}
PF17	95±0.04^{e}	5.5±0.05^{e}	14.7±0.06^{e}	1.98±0.07^{e}	0.506±0.06^{e}	86±0.05^{e}	3.3±0.06^{e}	11.4±0.07^{e}	1.54±0.08^{e}	0.371±0.05^{e}
PF23	100+0.02^{f}	6.0+0.01^{f}	15.1+0.03^{f}	2.10± 0.01^{f}	0.520± 0.01^{f}	90± 0.01^{f}	4.1± 0.03^{f}	12.1± 0.01^{f}	1.61± 0.01^{f}	0.393± 0.03^{f}
M.phaseolina	40± 0.01^{a}	2.6± 0.01^{a}	08.0± 0.02^{a}	1.11± 0.01^{a}	0.098± 0.02^{a}	40± 0.01^{a}	1.0± 0.02^{a}	3.0 ± 0.01^{a}	0.60± 0.01^{a}	0.118± 0.01^{a}
PF17+*M.phaseolina*	85±0.09^{c}	4.8±0.08^{c}	12.2±0.03^{c}	1.52±0.04^{c}	0.215±0.06^{c}	78±0.07^{c}	2.8±0.06^{c}	7.9±0.05^{c}	1.19±0.01^{c}	0.202±0.02^{c}
PF23+*M.phaseolina*	90± 0.02^{d}	5.0± 0.02^{d}	13.0± 0.01^{d}	1.61± 0.03^{d}	0.291± 0.01^{d}	80± 0.03^{d}	3.0± 0.03^{d}	9.0± 0.03^{d}	1.22± 0.02^{d}	0.217± 0.03^{d}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replications

Table 5: Influence of *Pseudomonas* treatment on growth of sunflower plants in both absence and presence of *M. phaseolina*, under non-saline (0 mM) and saline (125 mM NaCl) conditions (in pot study)

	Non- saline conditions (0 mM NaCl)						**Saline conditions (125 mM NaCl)**					
Treatments	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (cm)**	**Head diameter (cm)**	**Seed Yield (g/pot)**	**RAS/RT**	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (cm)**	**Head diameter (cm)**	**Seed yield (g/pot)**	**RAS/RT**
Unbacterized seeds	08+0.03[b]	50.3+0.02[b]	01.51+0.02[b]	06.32+0.03[b]	10.5+0.03[b]	0.553+0.02[b]	06.31+0.03[b]	45.1+0.04[b]	01.31+0.02[b]	05.01+0.03[b]	09.0+0.05[b]	0.321+0.03[b]
PF17	09.21+0.02[d]	57+0.03[d]	02.12+0.01[d]	07.2+0.01[d]	12.01+0.01[d]	1.180+0.01[d]	07.8+0.02[d]	54.4+0.02[d]	01.70+0.03[d]	06.7+0.05[d]	12.1+0.06[d]	1.001+0.04[d]
PF23	10.95+0.04[f]	62.1+0.05[f]	02.39+0.03[f]	08.1+0.04[f]	16.1+0.04[f]	1.231+0.03[f]	09.24+0.05[f]	59.8+0.06[f]	02.18+0.04[f]	07.45+0.04[f]	13.5+0.06[f]	1.021+0.04[f]
M. phaseolina	06.17+0.05[a]	35.9+0.04[a]	0.89+0.04[a]	05.1+0.03[a]	07.11+0.05[a]	0.319+0.05[a]	05.22+0.06[a]	32.6+0.05[a]	0.68+0.05[a]	04.2+0.05[a]	05.0+0.07[a]	0.359+0.05[a]
PF17+ *M.phaseolina*	08.34+0.01[c]	54.7+0.01[c]	01.97+0.02[c]	06.78+0.02[c]	11.79+0.02[c]	1.164+0.04[c]	07.11+0.04[c]	52.3+0.03[c]	01.67+0.07[c]	05.45+0.02[c]	10.47+0.03[c]	1.011+0.03[c]
PF23+*M.phaseolina*	09.94+0.02[e]	59.9+0.03[e]	02.01+0.05[e]	07.5+0.04[e]	13.7+0.06[e]	1.189+0.06[e]	08.54+0.07[e]	56.2+0.04[e]	01.82+0.06[e]	07.01+0.06[e]	11.9+0.04[e]	0.949+0.04[e]

Results are the mean of 05 replicates. Means in the columns followed by same letters indicates no significant difference (P = 0.05) by Duncan's Multiple Range Test. Five samples were analyzed for each replication, and each treatment consisted of five replications

4.11 PGP and Biocontrol Traits of PF23

4.11.1 Chemical Mutagenesis for Developing EPS-Defective Mutant

Of the 100 mutant clones, 6 were identified as defective for EPS production. Two of these mutants had stable mutations. EPS-defective stable mutant $PF23^{EPS-}$ showed maximum reduction of 86 % in production of EPS in comparison with wild strain PF23.

4.11.2 Salinity Stress Assay

As mentioned earlier PF23 could tolerate salinity level up to 2,000 mM (12 %). Salt shock with 100 mM NaCl did not affect the growth, but higher osmotic stress of 500, 1,000, and 1,500 brought significant reduction in OD by 32, 48, and 68 %, respectively, in comparison with non-stress conditions (0 mM NaCl) (Figure 11 a). No growth was observed above 2,000 mM salt concentration. On the other side EPS defective strain $PF23^{EPS-}$ displayed 80 % reduction in growth in presence of 200 mM NaCl, suggesting it to be a non-salt tolerating strain.

4.11.3 Effect of Saline Stress on EPS Production

Increase in osmotic stress or increase in salinity, brought increment in the EPS production up to a certain limit. In stress tolerating strain PF23, there was increase in EPS production by about 26, 38, and 66 % at salinity level of 1,000, 1,500, and 2,000, respectively (Table 6). EPS defective mutant $PF23^{EPS-}$ showed maximum EPS production at 0 mM NaCl (0.109 g/l) which drastically reduced above 100 mM NaCl, and finally got diminished beyond 200 mM NaCl, respectively.

4.11.4 Composition of EPS

Analysis of EPS constituents by thin layer chromatography revealed differences in the sugar components of salinity tolerant strain PF23 under non-stressed and stressed conditions. Under normal conditions (0 mM NaCl) glucose (Rf 0.42) was present as the saccharide unit in the EPS hydrosylate, whereas EPS obtained under salt stress was composed of glucose (Rf 0.42), galactose (Rf 0.37), rhamnose (Rf 0.74), mannose (Rf 0.46), and trehalose (Rf 0.32). Carbohydrate content of EPS progressively increased from 126 to152 µg /mg^{-1} with increase in salinity from 0 to 2,000 mM. EPS-defective strain PF23^{EPS-} contained only glucose as its saccharide unit both under control and maximum stress conditions (0-200 mM) and there was steep reduction in carbohydrate content from 20 µg/mg^{-1} to 5 µg/mg^{-1} under same conditions (Table 6).

4.11.5 Fourier Transform Infrared Spectroscopy

The FTIR spectrum of *Pseudomonas* EPS under different salt concentrations was analyzed and absorption bands gave typical polymeric structure of the carbohydrate (Figure 22). The stretching in the region 3296.7 cm^{-1} to 3388.9 cm^{-1} at different salinity was observed which represented the stretching vibration of the hydroxyl groups of carbohydrate. The absorption band at 2934.7 cm^{-1} to 2968 cm^{-1} represent the C-H stretching of methyl and methylene group. The absorption band found in the region 1617.2 cm^{-1} to 1655.4 cm^{-1} usually represents the stretching vibration of enol and amide group in the EPS at diverse NaCl concentration. The enol and amide group was absent in EPS obtained from 2000 mM NaCl as absorption band of 1617.2 cm^{-1} to 1655.4 cm^{-1} was absent in it. The stretching of C=O group was indicated by absorption band at 1617.2 cm^{-1} to 1655.4 cm^{-1}. Similarly, the peaks at 1401.6 –1423.3 cm^{-1} could be assigned to >C=O stretch of the COO− groups and C=O bond from COO^{-} groups. The absorption bands at 1013 cm^{-1} to 1086.2 cm^{-1} represent the –C–O stretching vibration. In the anomeric region, absorption band at 764.6 to 785.7 cm^{-1} revealed the possible presence of alpha glycosidic linkages. On the contrary, presence of band at 838–8870 cm^{-1} indicated that there could be β - glycosidic linkage in the EPS. The exclusive absorption bands observed at 724 cm^{-1} could be attributed to C-O-C bending vibration from the EPS obtained at 2000 mM NaCl.

4.11.6 Scanning Electron Micrographs

The SEM of dried EPS obtained at 0 mM revealed rough, wrinkled surface with depressions and grooves, indicating availability of probable binding sites for ions (Figure 23 a). This structure of EPS showed exact resemblance with the EPS obtained at 100 mM NaCl (Figure 23 b). As the level of salinity (500 mM) increased, Na^{+} gets biosorbed on the pores or grooves of EPS, and appears as white encrustations (Figure 23c). Photomicrography of EPS under saline conditions appears as large masses of dense filaments with small pores, randomly mixed to form three dimensional clump network (Figure 23 d; e and f). However, EPS obtained from PF23 under non - saline conditions at 0 mM NaCl appear to be smooth, glittering, spherical or ovoid exhibiting compact structure (Figure 23a). It can be observed from the photomicrographs that the EPS particles obtained under stressed conditions (500- 2000 mM NaCl) appear polyhedral in shape with wrinkled surface, while EPS obtained from 0 mM NaCl are ovoid to spherical shape. Further, it can be noted that the particles of EPS under saline conditions are smaller in size than under non- saline state. Thus, increase in saline stress affects shape and decreases the particle size of EPS.

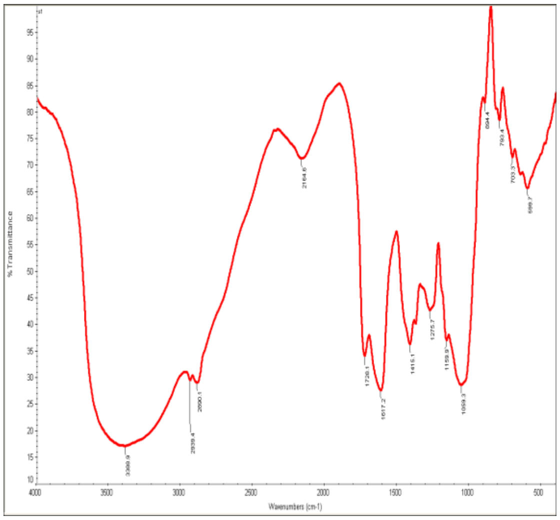
4.11.7 *In vitro* Biocontrol Activity of EPS

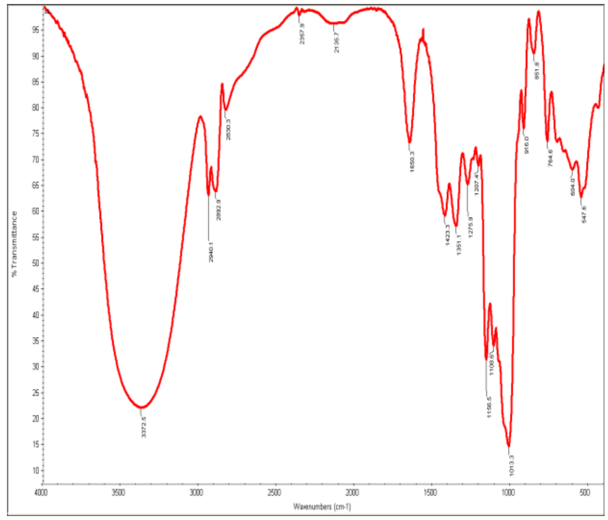
EPS obtained from PF23 at 100, 200, 300, 400, and 500 mM NaCl displayed strong antagonistic activity against *M. phaseolina* and resulted in 79, 72, 64, 55, and 41% reduction in growth, respectively, in comparison with control. EPS obtained at 0 mM (control) antagonized *M. phaseolina* by 84%. No biocontrol was displayed by mutant strains under saline conditions. Post interaction abnormalities in fungal mycelium by purified EPS obtained at 0 mM showed displayed halo formation in mycelium (Figure 24a). However, purified EPS at 200, 400 and 500 mM NaCl showed deformities and curling of fungal mycelium followed by degradation and lysis of fungal cell wall (Figure 24).

4.11.8 SA Production and Extraction

Qualitative and quantitative analysis suggested that the EPS producing strain $PF23^{EPS+}$ displayed SA production up to 500 mM NaCl. The SA produced by $PF23^{EPS+}$ was confirmed by the blue bands that appeared on pre-coated silica gel plate, when viewed under UV illumination. A fluorescent blue spot in the samples co-migrated with standard of SA, which also displayed same fluorescence. The R_f value of SA (0.61) produced by the $PF23^{EPS+}$ strain at different NaCl concentrations matched with the R_f value (0.61) of the standard SA. Thickness of the band obtained at 0 and 100 mM NaCl showed exact resemblance with control SA, however, further increase in salinity resulted in thinner bands (Figure 25). No SA production was observed beyond 500 mM by $PF23^{EPS+}$.

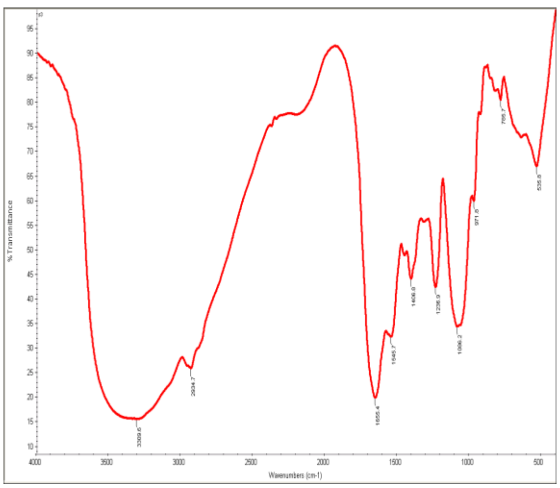
Quantitative analysis further authenticated qualitative results. Under control conditions (at 0 mM NaCl) bacterial strain produced 6.14 mg ml^{-1} of SA, which was significantly similar to the SA obtained at 100 mM salinity (6.12 mg). However, there was 16.2%, 70.5% and 90% reduction in SA production with progressive increase in salinity levels to 200 mM, 300 mM and 400 mM NaCl, respectively (Table 7).

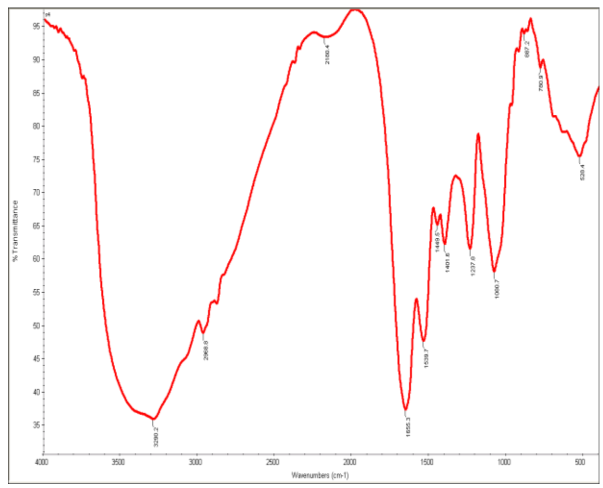




0 mM NaCl

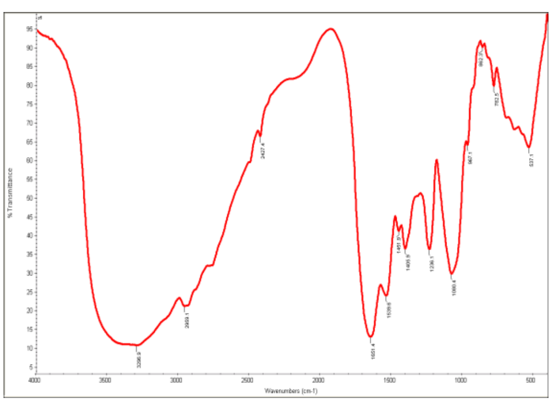
100 mM NaCl

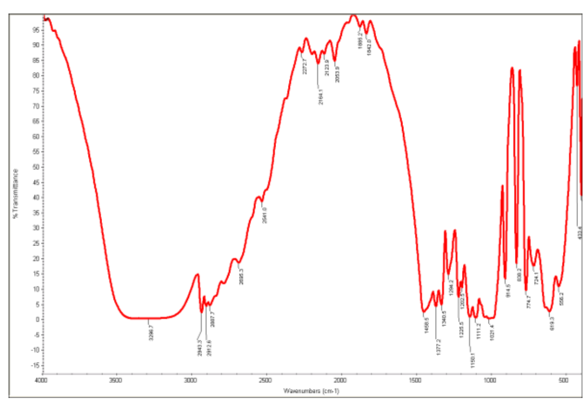




500 mM NaCl

1000 mM NaCl

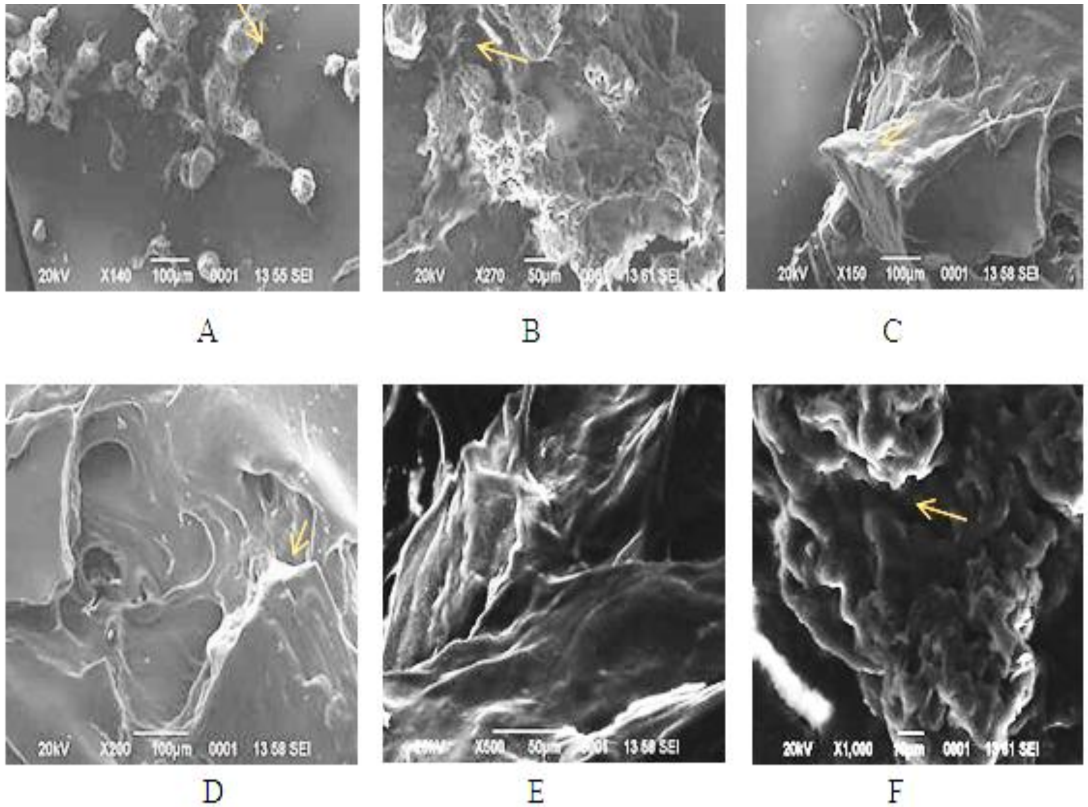




1500 mM NaCl

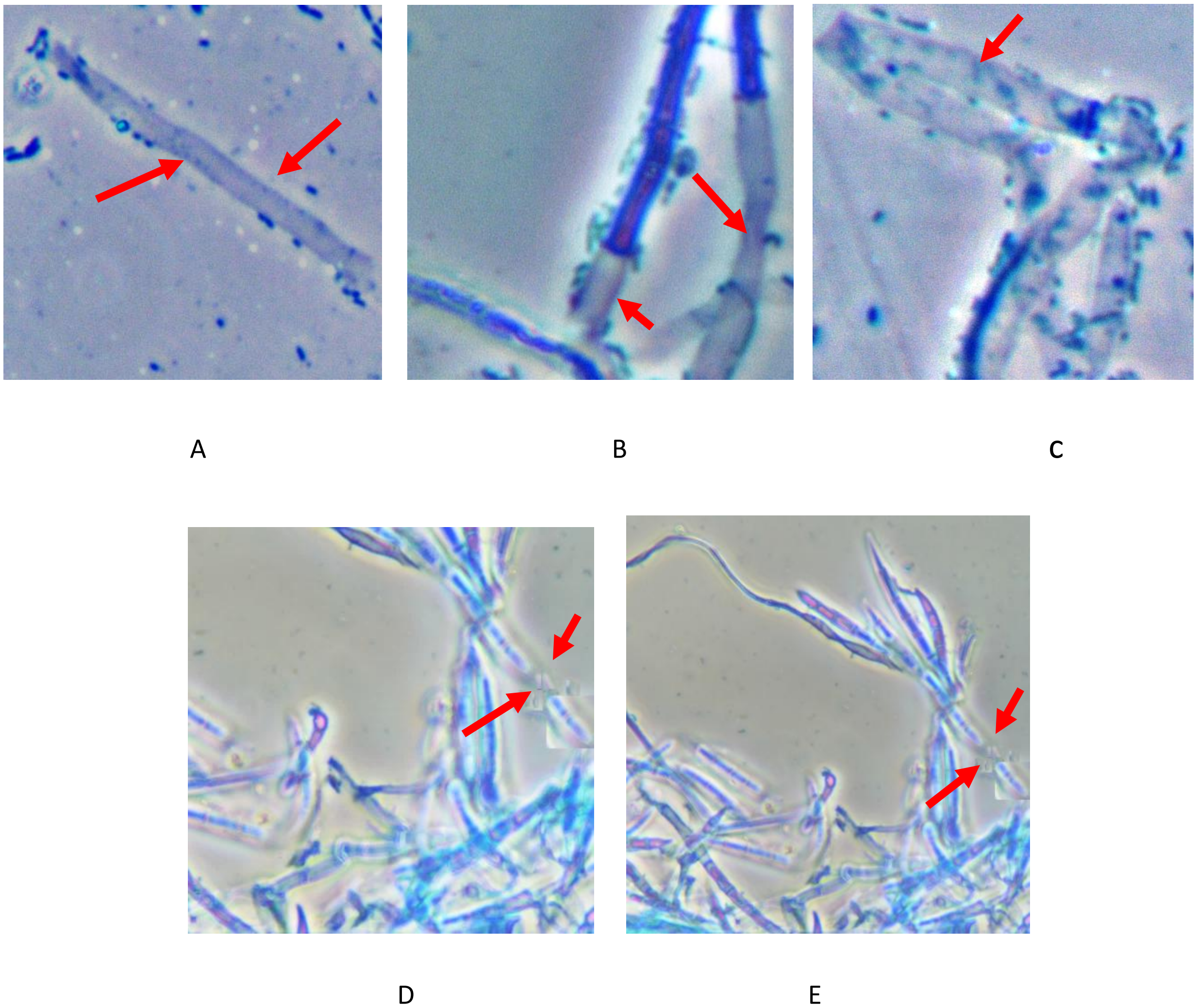
2000 mM NaCl

Figure 22: FTIR analysis of EPS at diverse NaCl concentrations



A: 0 mM NaCl; B: 100 mM NaCl; C: 500 mM NaCl; D: 1000 mM NaCl; E: 1500 mM NaCl; F: 2000 mM NaCl

Figure 23: SEM analysis of EPS obtained under non – saline and saline conditions



A and B: Halo formation by EPS at 0 and 100 mM
C: Curling of hyphae by EPS at 300 mM
D and E: deformities and lysis of hyphae by EPS at 400 and 500 mM

Figure 24: Post interaction morphological changes in *M. phaseolina* due to purified EPS

Table 6 : Effect of saline stress on EPS production

Salt stress (mM)	Dry weight of EPS (g/l)	EPS components	Carbohydrate in EPS (µg/mg^{-1})
0	0.801±0.01[a]	Glucose	126±0.02[a]
100	0.811±0.02[a]	Glucose, galactose	131±0.01[b]
500	0.901±0.03[ab]	Glucose, galactose	133±0.03 [b]
1000	1.010±0.01[b]	Glucose, rhamnose	138 ±0.02[c]
1500	1.102±0.02[c]	Glucose, rhamnose, trehalose	145±0.01 [d]
1700	1.210±0.01[cd]	Glucose, mannose, rhamnose, trehalose	149±0.03 [de]
2000	1.323±0.03[d]	Glucose, mannose, rhamnose, trehalose	152±0.02[e]

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replicates

EPS defective mutant PF23^{EPS-} demonstrated 0.03 mg ml^{-1} SA production and very bleached band of SA at 0 mM NaCl. Whereas, further increase in salinity brought complete loss in SA thereby suggesting suppression of SA synthesis in mutant strain.

4.11.9 High Performance Liquid Chromatography

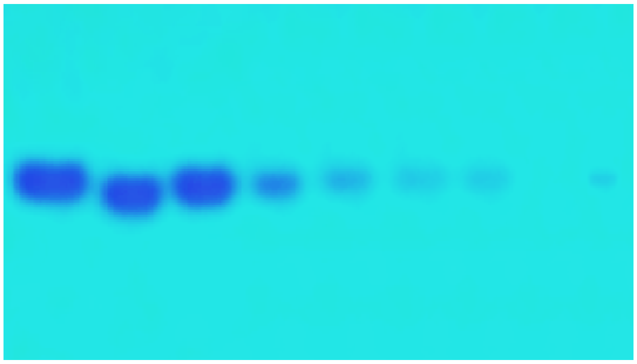
HPLC analysis revealed single peak up to 500 mM NaCl concentration for PF23^{EPS+} at different salinity levels that resembled to standard SA in respect to its retention time (4.502 min). However, no peak was observed in mutant strain at salinity levels except above 0 mM NaCl (Figure 26).

4.11.10 Antagonistic activity of SA against *M. phaseolina*

Biocontrol ability of the strain PF23^{EPS+} was maintained up to 500 mM salinity but thereafter inhibitory activity ceased. SA obtained from PF23^{EPS+} at 100, 200, 300, 400 and 500 mM NaCl displayed strong antagonistic activity against *M. phaseolina* and resulted in 73.4%, 60.3%, 50.8%, 39.1%, and 19.01 % reduction in growth, respectively, in comparison to control. SA acquired from PF23^{EPS+} and PF23^{EPS-} at 0 mM NaCl (control) antagonized *M. phaseolina* by 75.02% and 14.1 %, respectively. Whereas, no antagonism was shown by the mutant strain with increase in salinity (Table 7).

4.11.11 Post Interaction Abnormalities in Fungal Mycelium

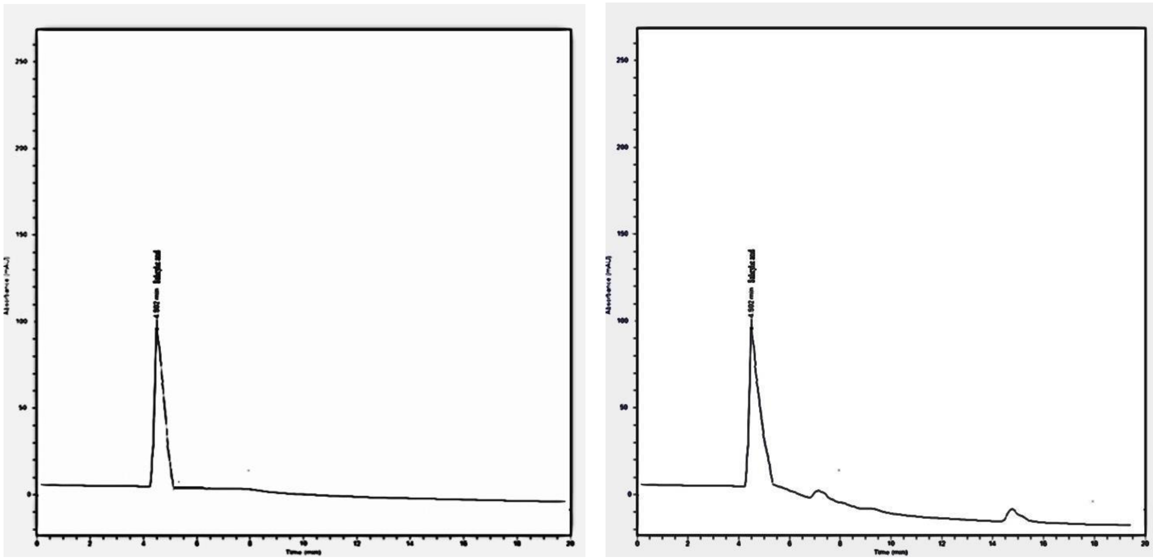
SA obtained from PF23^{EPS+} caused halo formation, mycelial deformities, hyphal tip perforation and degradation of *M. phaseolina.* Sclerotial development in *M. phaseolina* was also arrested as proven by the electron micrographs (Figure 27).



a b c d e f g h i

a: Standard SA
b: SA produced by PF23^{EPS+} (0 mM NaCl)
c: SA produced by PF23^{EPS+} (100 mM NaCl)
d: SA produced by PF23^{EPS+} (200 mM NaCl)
e: SA produced by PF23^{EPS+} (300 mM NaCl)
f: SA produced by PF23^{EPS+} (400 mM NaCl)
g: SA produced by PF23^{EPS+} (500 mM NaCl)
h: No SA produced by PF23^{EPS+} (600 mM NaCl)
i: SA produced by PF23^{EPS-} (0 mM NaCl)

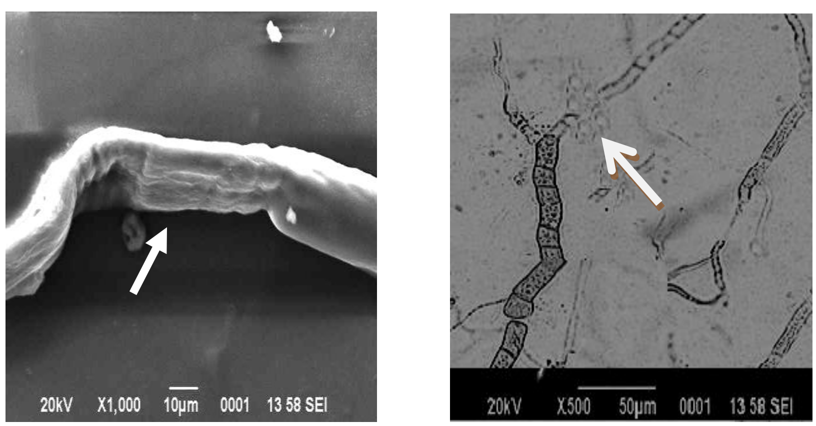
Figure 25: Thin layer chromatograph showing SA production by PF23^{EPS+} and its mutant under different NaCl concentrations



a b

(a) Standard SA **(b) Purified SA**

Figure 26: HPLC chromatograms of SA



a **b**

a: Deformities in fungal mycelium b: Digestion of fungal cell wall

Figure 27 : Post interaction morphological changes in *M. phaseolina* due to purified SA

Table 7: Effect of salinity on SA production and antagonistic activity against *M. phaseolina*

NaCl (mM)	SA production by PF23 $^{EPS+}$	% of Antagonism
0	6.14 ± 0.08^{e}	75.12 ± 0.01^{e}
100	6.12 ± 0.04^{e}	73.40 ± 0.02^{e}
200	5.28 ± 0.02^{d}	60.31 ± 0.03^{d}
300	3.60 ± 0.06^{c}	50.82 ± 0.04^{c}
400	1.11 ± 0.07^{b}	39.10 ± 0.06^{b}
500	0.05 ± 0.03^{a}	19.01 ± 0.08^{a}
600	-	-

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replication

4.12 *In vivo* Pot Study

In pot study best results were obtained when amalgamation of EPS along with SA was coated on sunflower seeds. There was significant increase in plant growth parameters observed both under saline and non-saline conditions in presence or absence of pathogen when amalgam of purified SA and EPS was dressed on sunflower seeds in comparison to individual pelleting of SA and EPS. Seed treatment with amalgamation enhanced seed yield by 134% and 152% respectively, under non-saline and saline conditions in comparison to control (untreated seeds). Seed dressing with SA along with PF23^{EPS+} brought enhancement in root length, shoot length, dry weight and seed yield by 116.6%, 44.44%, 135.8% and 100% respectively, in comparison to unbacterized seeds (positive control) under saline conditions (Table 8). Purified EPS when applied on the seeds brought enhancement in seed yield by 100% and 122% under non-saline and saline conditions respectively (Figure 28).

Seed coating with PF23^{EPS+} cells brought 53.3% and 50% enhancement in seed yield under non-saline and saline conditions respectively, in comparison to unbacterized seeds. However, purified SA from same strain brought enhancement in seed yield by 73.4% and 78.7%, under non-saline and saline conditions respectively, in comparison to untreated seeds. PF23^{EPS-} displayed significantly similar results as obtained by control (untreated seeds), both under saline and non-saline conditions. Seed pelleting with purified SA showed significantly similar enhancement in growth attributes in comparison to the set receiving treatment of standard SA, both under saline and non-saline conditions, in infested as well as non-infested conditions. No SA was obtained from EPS defective strain under saline conditions, thereby confirming the suppression of SA along with EPS in defective strain. Seeds infested with *M. phaseolina* showed 79 % and 81 % disease incidence in non-saline and saline soil, respectively. Treatment of seeds with PF23^{EPS+} resulted in 71% and 63% reduction of disease incidence in comparison with non-bacterized seeds (control) under non-saline and saline conditions, respectively, whereas seed pelleting with purified SA brought 75% and 67% reduction in disease incidence respectively. This was significantly similar to the sets receiving treatment with standard SA. Combination of PF23^{EPS+} along with SA displayed best results in respect of suppressing charcoal rot incidence in sunflower. The combination of EPS + SA brought 79% and 71% reduction of disease incidence in sunflower under non-saline and saline conditions, respectively. However, EPS-defective strain brought reduction of disease incidence by 23 and 17% under non-saline and saline conditions, respectively.

In presence of pathogen (*M. phaseolina*), strain PF23^{EPS+} caused 100% and 140% enhancement in seed yield under non-saline and saline conditions, respectively, in comparison with untreated seeds. Purified SA, in presence of pathogen, enhanced seed yield by 129% and 180% in comparison to negative control under non-saline and saline conditions, respectively. The combination of SA and EPS (in presence of pathogen) brought enhancement in seed yield by 257% and 360% respectively, in comparison to *M. phaseolina* infested seeds under non-saline and saline conditions in comparison to negative control. The increase in seed yield and other plant growth parameters by purified SA treatment was significantly similar to the set receiving treatment with standard SA. But, the mutant was ineffective in controlling the pathogen under saline conditions. EPS defective strain was ineffective in suppressing disease incidence under saline conditions.

a b c d

A: a: EPS ; b: SA ; c: untreated seeds (control); d: *M. phaseolina* (negative control)

a b c d

B: a: EPS ; b: SA ; c: untreated seeds (control); d: *M. phaseolina* (negative control)

Figure 28: *In vivo* (pot) study under saline conditions (A: planted and B: uprooted)

Table 8: Influence of diverse treatments on growth of sunflower plants in both absence and presence of *M. phaseolina* under non-saline (control) and saline (125 mM NaCl) conditions in pot study

	Non saline conditions (control)					**Saline Stress (125 mM)**				
Treatments	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (g)**	**Head diameter (cm)**	**Seed yield/pot (g/pot)**	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (g)**	**Head diameter (cm)**	**Seed yield/pot (g/pot)**
Untreated seeds	08.00± 0.02^{b}	50.3± 0.09^{b}	01.51± 0.08^{b}	06.32± 0.01^{b}	10.50± 0.02^{b}	06.31± 0.08^{b}	45.1± 0.09^{b}	01.31± 0.01^{b}	05.01 ± 0.03^{b}	09.00± 0.07^{b}
PF23^{EPS+}	10.95± 0.04^{d}	62.1± 0.08^{d}	02.39± 0.06^{d}	08.10 ± 0.02^{d}	16.10± 0.08^{d}	09.24± 0.02^{c}	59.80± 0.03^{c}	02.18± 0.04^{d}	07.45± 0.05^{d}	13.50± 0.06^{d}
PF23^{EPS-}	08.50± 0.08^{b}	55.6± 0.07^{b}	01.58± 0.07^{b}	06.71± 0.03^{b}	11.70± 0.06^{b}	06.32± 0.07^{b}	46.9 ± 0.04^{b}	01.34± 0.02^{b}	05.12± 0.03^{b}	09.30± 0.02^{b}
M. ph.	06.17± 0.05^{a}	35.9± 0.06^{a}	0.89± 0.09^{a}	05.10± 0.06^{a}	07.11± 0.02^{a}	05.22± 0.04^{a}	32.6± 0.08^{a}	0.68± 0.06^{a}	04.20± 0.04^{a}	05.00± 0.03^{a}
PF23^{EPS+} + *M. ph.*	09.94± 0.07^{c}	59.9± 0.05^{c}	02.01± 0.03^{c}	07.50± 0.08^{c}	13.70± 0.03^{c}	08.54 ± 0.08^{c}	56.2± 0.05^{c}	01.82± 0.07^{c}	07.01± 0.09^{c}	11.90± 0.02^{c}
PF23 $^{EPS-}$+ *M. ph.*	08.30± 0.06^{b}	53.1± 0.04^{b}	1.53± 0.04^{b}	06.50± 0.09^{b}	11.30± 0.06^{b}	05.32± 0.06^{a}	33.7± 0.05^{a}	0.72± 0.04^{a}	04.50± 0.01^{a}	05.30± 0.02^{a}
Purified EPS	15.31± 0.07^{h}	67.07± 0.01^{h}	05.87± 0.03^{h}	13.47± 0.07^{h}	21.71± 0.05^{h}	14.29± 0.05^{h}	65.07± 0.01^{h}	04.53± 0.04^{h}	11.97± 0.07^{h}	19.72± 0.05^{h}
Purified EPS + *M. ph.*	15.09± 0.06^{h}	66.97± 0.02^{h}	05.53± 0.04^{h}	13.16± 0.05^{h}	20.79± 0.04^{h}	14.97± 0.03^{h}	65.97± 0.02^{h}	04.27± 0.03^{h}	11.26± 0.05^{h}	19.41± 0.04^{h}
Purified EPS + PF23^{EPS+}	16.51± 0.05^{i}	68.49± 0.03^{i}	06.41± 0.05^{i}	14.97± 0.07^{i}	22.62± 0.05^{i}	15.62± 0.05^{i}	66.27± 0.03^{i}	05.03± 0.04^{i}	12.97± 0.07^{i}	20.12± 0.05^{i}
Purified EPS+PF23^{EPS+} + M.ph	15.99± 0.04^{i}	67.72± 0.04^{i}	06.03± 0.06^{i}	14.06± 0.05^{i}	22.09± 0.04^{i}	15.07± 0.04^{i}	66.26± 0.04^{i}	04.81± 0.05^{i}	12.46± 0.05^{i}	20.09± 0.04^{i}
Purified EPS + PF23^{EPS-}	15.28± 0.03^{h}	67.01± 0.05^{h}	05.69± 0.07^{h}	13.61± 0.07^{h}	21.29± 0.05^{h}	15.02± 0.05^{h}	65.03± 0.05^{h}	04.31± 0.06^{h}	11.61± 0.07^{h}	19.43± 0.05^{h}
Purified EPS + PF23^{EPS-} + *M. ph.*	14.97± 0.02^{h}	66.97± 0.06^{h}	05.51± 0.08^{h}	13.36± 0.05^{h}	20.51± 0.04^{h}	14.01± 0.04^{h}	65.01± 0.06^{h}	04.11± 0.07^{h}	11.16± 0.05^{h}	19.31± 0.04^{h}
Purified SA	12.21± 0.02^{f}	64.27± 0.02^{f}	03.04± 0.05^{f}	10.65± 0.02^{f}	18.21± 0.01^{f}	11.76± 0.04^{e}	62.01± 0.03^{d}	02.39± 0.07^{e}	08.02± 0.08^{f}	16.09± 0.05^{f}
Purified SA +*M. ph.*	11.10± 0.07^{e}	60.02± 0.01^{e}	02.02± 0.07^{e}	09.13± 0.03^{e}	15.23± 0.08^{e}	10.49± 0.06^{d}	59.24± 0.08^{e}	01.76±0.08^{f}	07.48± 0.04^{e}	14.08 ± 0.08^{e}
Purified SA+ PF23^{EPS+}	14.56± 0.05^{g}	66.34± 0.07^{g}	3.82± 0.08^{g}	12.56± 0.09^{g}	19.67± 0.04^{g}	13.67± 0.09^{g}	64.75± 0.01^{g}	3.09± 0.07^{g}	10.30± 0.09^{g}	17.59± 0.07^{g}
Purified SA+PF23^{EPS+}+*M. ph.*	14.01± 0.04^{g}	65.51± 0.06^{g}	3.20± 0.01^{g}	12.09± 0.02^{g}	19.02± 0.06^{g}	13.12± 0.08^{g}	64.01± 0.08^{g}	2.81± 0.07^{g}	10.01± 0.07^{g}	17.01± 0.07^{g}
Purified SA+PF23^{EPS-}	11.98± 0.02^{f}	63.01± 0.02^{f}	02.84± 0.05^{f}	10.51± 0.02^{f}	07.99± 0.01^{f}	11.21± 0.04^{e}	61.01± 0.03^{d}	02.19± 0.07^{e}	07.98± 0.08^{f}	16.00± 0.05^{f}
Purified SA+PF23^{EPS-}+*M. ph.*	11.00± 0.07^{e}	60.03± 0.02^{f}	01.97± 0.07^{e}	09.01± 0.03^{e}	15.02± 0.08^{e}	10.21± 0.06^{d}	59.01± 0.08^{e}	01.71±0.08^{f}	07.31± 0.04^{e}	14.00 ± 0.08^{e}
Purified SA+ purified EPS	17.31± 0.02^{j}	70.51± 0.02^{j}	07.87± 0.04^{j}	16.47± 0.07^{j}	24.56± 0.03^{j}	16.82± 0.03^{j}	67.65± 0.05^{j}	05.71±0.04^{j}	13.71±0.08^{j}	22.71±0.04^{j}
Purified SA+ purified EPS +*M. ph*	17.09± 0.02^{j}	69.07± 0.01^{j}	07.53± 0.03^{j}	15.96± 0.05^{j}	24.02± 0.07^{j}	16.11± 0.06^{j}	67.01± 0.04^{j}	05.10±0.06^{j}	13.08±0.07^{j}	22.01±0.03^{j}
Standard SA	12.54± 0.08^{f}	64.42± 0.05^{f}	03.12± 0.09^{f}	10.82± 0.01^{f}	18.43± 0.07^{f}	11.91± 0.09^{e}	59.45± 0.02^{e}	02.48±0.09^{e}	08.76± 0.03^{f}	16.19± 0.03^{f}
Standard SA+ *M. ph.*	11.23± 0.09^{e}	60.51± 0.07^{e}	02.08± 0.01^{e}	09.22± 0.02^{e}	15.54± 0.08^{e}	10.98 ± 0.08^{d}	62.18± 0.05^{d}	01.79±0.01^{f}	07.84± 0.08^{e}	14.11± 0.04^{e}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replication

4.13 Bioformulation Production and Field Trials

4.13.1 Physico-Chemical Properties of Carriers

Amongst all the tested carriers, talc showed maximum IMC (11.1%) and WHC (302%) respectively followed by charcoal (10.5% and 267%) and sawdust (9.5% and 285%). Coriander husk (6.76% and 230%), corn husk (5.65% and 210%) and coconut husk displayed 3.2% and 160% moisture content and water holding capacity respectively. The pH of all the carriers was near neutral (6.8 to 7.4). After six month of storage the IMC of the talc was maintained at 10.09 % which was found maximum amongst the tested carriers (Table 9).

4.13.2 Development of Carrier Based Inoculum

Amongst all the selected carriers, talc formulation served best, in supporting population of PF17 and PF23 both under stressed and non-stressed conditions. PF23 maintained population of 7.0 log cfu/g and 7.1 log cfu/g in talc formulation, under non stress and stress conditions after 180 days. However, PF17 population was 6.9 log cfu/g and 7.0 log cfu/g in talc formulation under non stress and stress conditions upto 180 days (Figure 29 a and b). It was observed that after talc, coriander husk was the most efficient carrier in supporting population density of bacterial cells both under stress and non-stress conditions, followed by sawdust.

In saw dust, PF23 maintained population of 6.3 log cfu/g and 6.0 log cfu/g under non - stress and stress conditions, respectively after 180 days. Although coconut husk maintained a constant cfu under stress and non-stress conditions for about 20 days; there was an abrupt decline in the cfu thereafter. Corn proved to be an average or intermediate for carrying bacterial cells supported population of 5.0 and 5.2 cells/g during stress and non-stress conditions.

4.13.3 *In vivo* Field Trails with Cell and CFCS Formulations

The results of field trials showed that formulation developed from cells and CFCS of PF23 (under non-stress conditions) suppressed disease incidence by 53.2% and 57.4% respectively, however, formulation developed from same cells and CFCS of PF23 under stress conditions, suppressed disease incidence by 55.9% and 59.4% respectively. Bioformulation developed from unstressed PF17 cells and its CFCS suppressed *M. phaseolina* infestation in sunflower by 45.8% and 47.1%. Whereas, formulation developed from stressed cells and CFCS of PF17 suppressed charcoal rot disease incidence in sunflower by 48.31% and 49.02% respectively.

Seed dressing with talc based formulation of unstressed PF17 cells brought enhancement in plant growth attributes including germination %, root length, dry weight and head diameter by 29%, 63.15%, 35% and 23.8% respectively, in comparison to control. Whereas stressed cells of PF17 enhanced germination %, root length, dry weight and head diameter by 33.7%, 78.64%, 52.5% and 33.3% respectively, in comparison to control (Table 10). Talc based formulation developed from stressed CFCS of PF17 brought enhancement in germination, root length, dry weight and head diameter by 35.5%, 82.3%, 60.01% and 44.44% respectively in comparison to control. Formulation developed from stressed and unstressed CFCS of PF17 enhanced seed yield by 93.8 and 79.38% in comparison to control, whereas formulation designed from stressed and unstressed cells of PF17 increased seed yield by 74.2 and 57.14% respectively in comparison to control.

Bioformulation designed from the stressed cells of PF23 enhanced germination, root length, dry weight and RAS/RT by 34.6%, 82.29%, 77.5% and 97.61% respectively in comparison to control seeds. Whereas formulation developed from stressed CFCS of PF23 enhanced growth attributes of sunflower including germination, root length, dry weight and RAS/RT by 37%, 94.73%, 85% and 97.70% respectively in comparison to control (Table 10). Formulation developed from stressed and unstressed CFCS of PF23 enhanced seed yield by 140 and 134%, however formulation developed from unstressed and stressed cells of PF23 enhanced seed yield by 132 and 133% respectively

Hence, it could be accounted that bio formulation developed from stressed CFCS of PF23 gave much better results in enhancing growth attributes of sunflower and suppressing disease incidence in comparison to cell based formulation.

4.13.4 Field Trails with Metabolite and Cell + Metabolite Formulation

Results of the field study highlighted that seeds sown in saline soil naturally infested with *M. phaseolina* showed 75% disease incidence. Seed dressing with formulation of purified EPS suppressed disease incidence by 66.7%, and formulation of SA obtained from the same strain caused 64.2% reduction of charcoal rot disease, however, the combination of EPS along with SA brought 70.2% reduction of charcoal rot disease incidence in sunflower plant that was significantly similar to the results of standard SA along with standard polysaccharide (dextran) (in combination). When formulation of standard dextrane and standard SA was applied as individual treatment it suppressed disease incidence of *M. phaseolina* by 65.1 % and 65.6 % respectively. When the formulation containing combination of EPS and SA along with PF23 cells was applied it suppressed disease incidence by 68.2% and 64% respectively.

The combination EPS with purified SA gave best results in enhancing plant growth parameters and suppressing the incidence of disease. Seed dressing with EPS formulation brought significant enhancement in growth parameters of sunflower similar to that of the standard polymer dextran. Bioformulation designed from EPS brought heightened germination, root length, dry weight, seed yield and RAS/RT ratio by 44.7%, 119.2%, 125%, 188.6% and 139.5% respectively, in comparison to untreated seeds (control) (Figure 30 a-d). However, formulation developed from purified SA displayed significant similarity to the set receiving treatment with standard SA formulation in presence of pathogen in salinized soil. SA formulation when applied on seeds brought enhancement in seed yield by 177.8 % in comparison to control (Table 10). Amalgamation of purified EPS and SA formulation brought maximum enhancement in germination, plant growth parameters and seed yield and displayed results almost similar to the treatment receiving combination of standard SA and dextran. Formulation containing blend of EPS and SA brought enhancement in seed yield and RAS/RT by 199.6% and 162.6% in comparison to control. Combined formulation also brought significant enhancement in seed yield and other plant growth parameters in comparison to mono formulation of EPS and SA (Figure 31). Combination of cells along with metabolite based formulation of EPS + PF23 and SA + PF23 was equally effective in enhanced seed yield by 195% and 180% respectively in comparison to control.

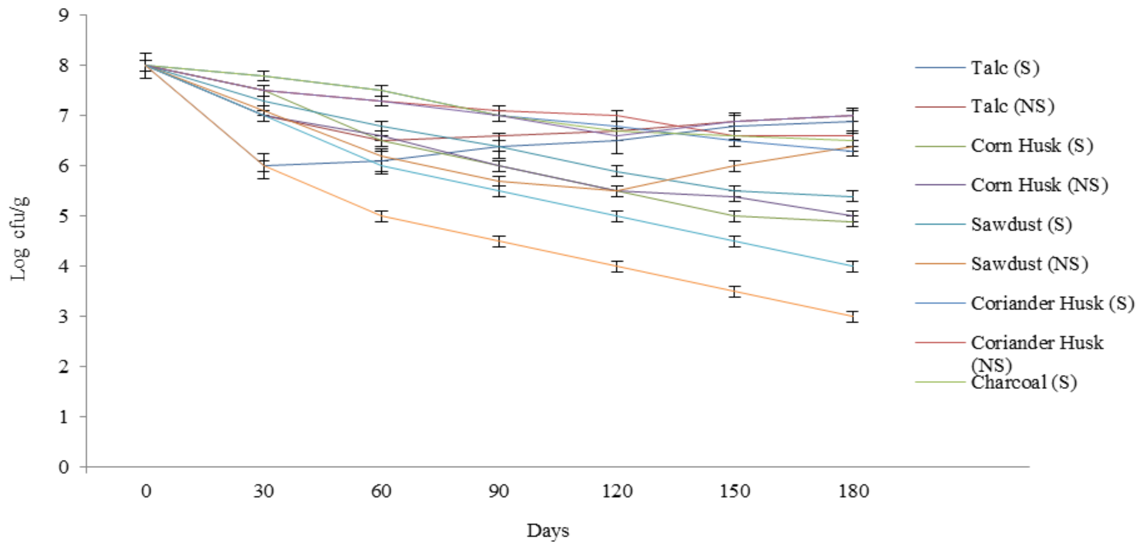
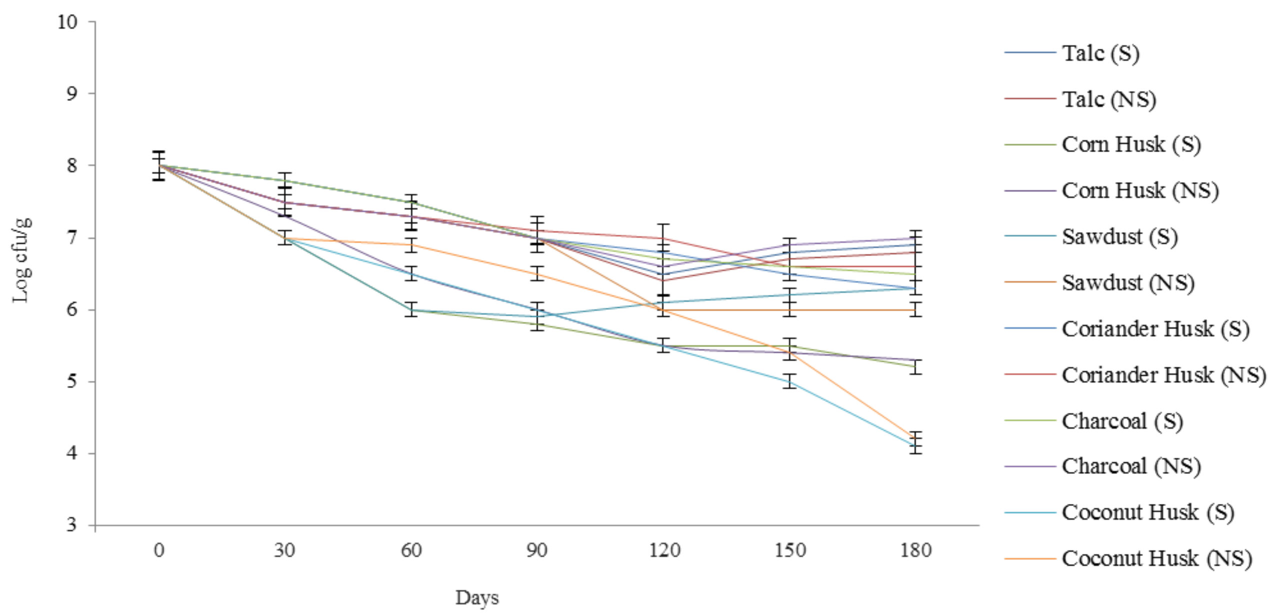


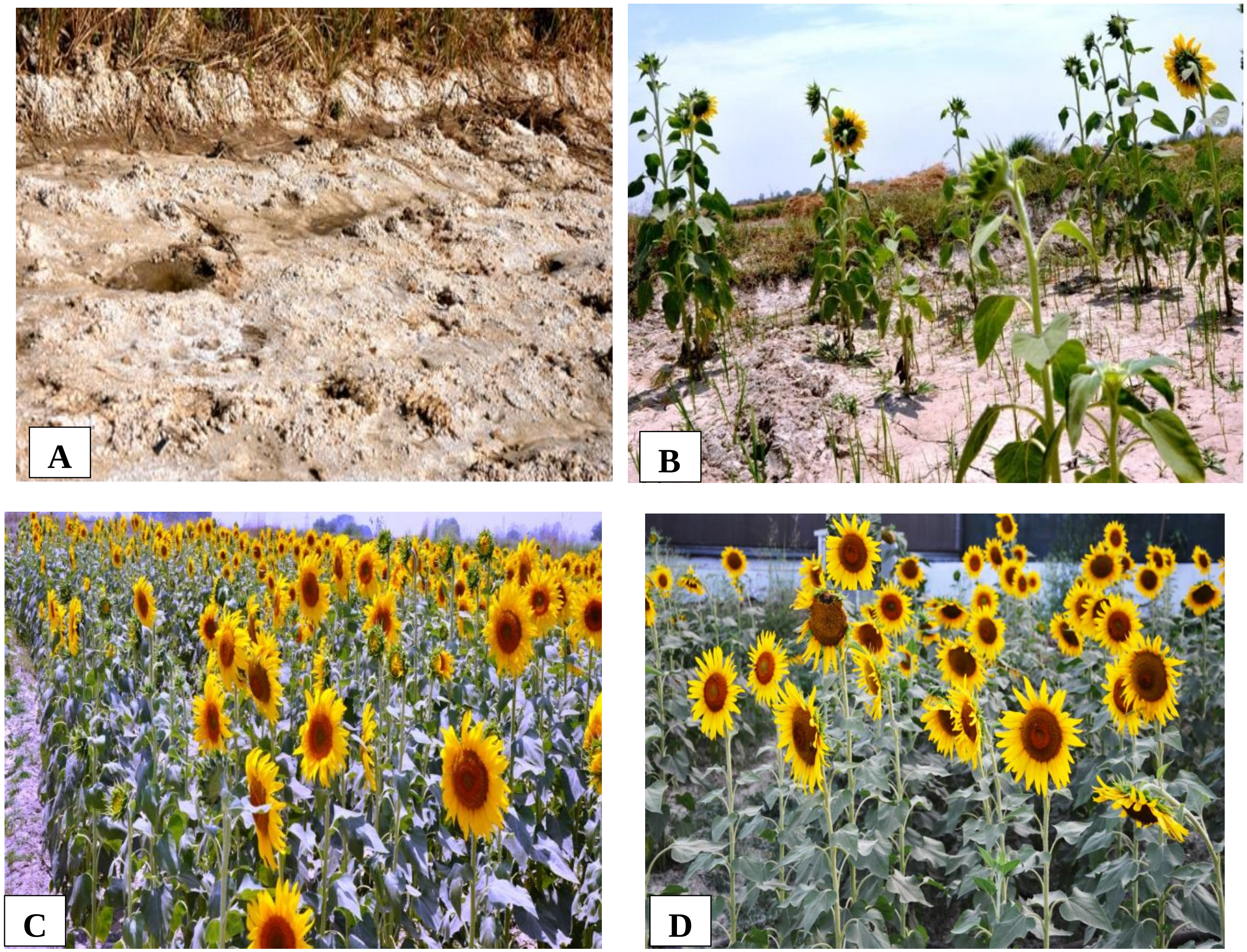
Figure 27a: Shelf life of PF17



b: Shelf life of PF23

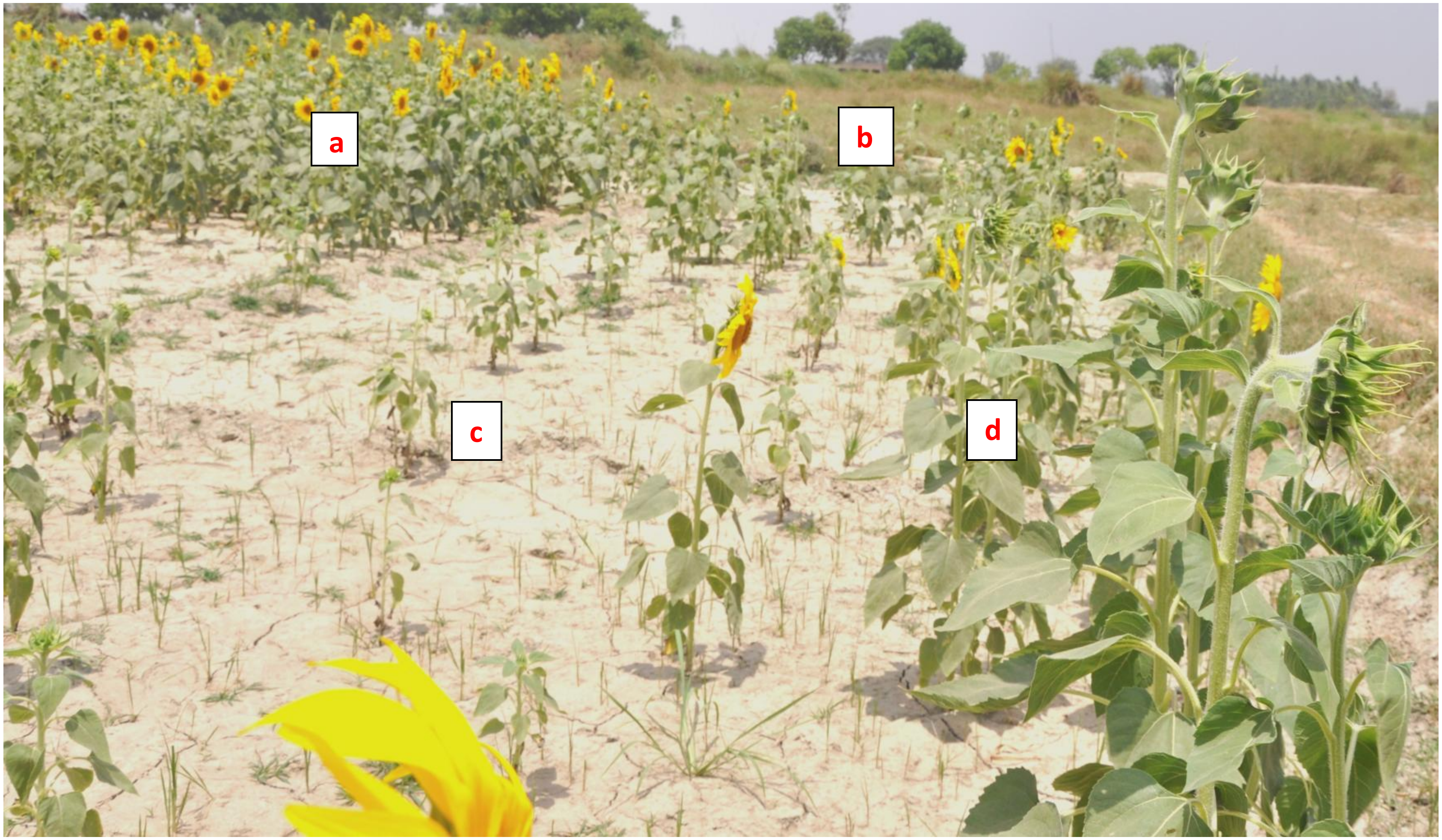
Figure 29: Shelf life of isolates in different carriers under stress and non-stress conditions

S: stress conditions; NS: Non stress conditions



A: Salinized field where experiment was carried; B: Untreated seeds ; C: EPS + SA formulation; D: EPS formulation

Figure 30: Plant growth promotion in salinized soil by applying diverse formulations



a: SA+EPSformulation; b: SA formulation; c: untreated; d:EPS formulation

Figure 31: Effect of diverse treatments on the growth of sunflower crop in saline soil

Table 9: Comparative assessment of different carriers

Carrier	**pH**	**Inherent moisture (%)**	**Water holding capacity (%)**	**Moisture content after 180 days (%)**
Talc	7.2 ± 0.05^{d}	11.1 ± 0.01^{f}	302 ± 0.02^{f}	10.09 ± 0.02^{f}
Charcoal	7.1 ± 0.02^{c}	10.5 ± 0.02^{e}	267 ± 0.01^{e}	8.82 ± 0.03^{e}
Sawdust	7.3 ± 0.01^{e}	9.56 ± 0.04^{d}	285 ± 0.02^{d}	7.14 ± 0.04^{d}
Coriander husk	6.8 ± 0.03^{b}	6.76 ± 0.03^{c}	230 ± 0.03^{c}	5.21 ± 0.02^{c}
Corn husk	6.9 ± 0.04^{a}	5.65 ± 0.02^{b}	210 ± 0.04^{b}	4.01 ± 0.03^{b}
Coconut husk	7.4 ± 0.01^{f}	4.92 ± 0.01^{a}	160 ± 0.03^{a}	3.02 ± 0.05^{a}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P =0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replicates

Table 10 : Effect of diverse bioformulations of PF17 and PF23 on growth of sunflower crop

Treatments	**Germination %**	**Root length (cm)**	**Shoot Length (cm)**	**Dry weight (g)**	**Head diameter (cm)**	**Chlorophyll (Chl a + Chl b) (mg/g)**	**Stem width (cm)**	**Leaf area (cm^2)**	**Seed yield (Kg/ Hectare)**	**RAS/RT**
Control	40.7± 0.05^{a}	19.2± 0.02^{a}	90.2± 0.01^{a}	4.0± 0.02^{a}	6.3 ± 0.01^{a}	0.0161± 0.05^{a}	2.22± 0.02^{a}	95.08± 0.05^{a}	96.7± 0.02^{a}	1.134± 0.05^{a}
PF17$_{(cells)\ unstressed}$	69.6± 0.04^{b}	31.1± 0.05^{b}	111.9± 0.04^{b}	5.4± 0.04^{b}	7.8± 0.05^{b}	0.0172± 0.01^{b}	2.50± 0.04^{b}	110.11± 0.01^{b}	154.3± 0.04^{b}	1.330± 0.01^{b}
PF17 $_{(cells)\ stressed}$	74.4± 0.05^{d}	34.3± 0.01^{c}	120.1± 0.06^{de}	6.1± 0.06^{c}	8.4± 0.07^{c}	0.0179± 0.04^{c}	2.65± 0.06^{c}	119.45± 0.03^{d}	168.7± 0.06^{c}	1.458± 0.05^{c}
PF17$_{(CFCS)\ unstressed}$	70.3± 0.02^{b}	32.8± 0.02^{b}	115.5± 0.05^{c}	5.6± 0.05^{b}	8.1± 0.02^{c}	0.0175± 0.06^{bc}	2.60± 0.06^{bc}	116.31± 0.02^{c}	173.8± 0.04^{d}	1.532± 0.04^{d}
PF17$_{(CFCS)\ stressed}$	76.2± 0.01^{d}	34.5± 0.06^{cd}	121.2± 0.02^{d}	6.4± 0.01^{cd}	9.1± 0.06^{d}	0.0191± 0.06^{e}	2.87± 0.04^{d}	135.54 ± 0.04^{e}	188.3± 0.06^{e}	1.660± 0.05^{e}
PF23$_{(cells)\ unstressed}$	70.2± 0.04^{b}	32.4± 0.04^{b}	116.9± 0.04^{c}	6.6± 0.06^{b}	9.0± 0.04^{b}	0.0186± 0.05^{d}	3.02± 0.05^{e}	139.01± 0.06^{f}	222.8± 0.04^{f}	2.234± 0.02^{f}
PF23 $_{(cells)\ stressed}$	75.3± 0.05^{d}	34.9± 0.05^{d}	119.5± 0.03^{d}	7.1± 0.05^{de}	9.3± 0.05^{d}	0.0190± 0.04^{e}	4.17± 0.06^{f}	142.91± 0.05^{g}	225.8± 0.05^{g}	2.241± 0.04^{g}
PF23$_{(CFCS)\ unstressed}$	72.4± 0.04^{c}	33.5± 0.01^{c}	118.4± 0.05^{d}	6. 9± 0.04^{d}	9.5± 0.07^{c}	0.0193± 0.06^{e}	4.39± 0.05^{g}	144.01± 0.06^{gh}	227.1± 0.06^{h}	2.236± 0.06^{f}
PF23$_{(CFCS)\ stressed}$	77.7± 0.05^{e}	36.5± 0.04^{e}	122.4± 0.02^{e}	7.4± 0.06^{e}	10.2± 0.04^{e}	0.0196± 0.05^{e}	4.87± 0.05^{h}	146.09± 0.05^{h}	231.5± 0.02^{i}	2.242± 0.02^{g}
EPS $_{(Formulation)}$	85.2± 0.02^{f}	42.1± 0.05^{f}	131.8± 0.04^{f}	9.0± 0.06^{f}	14.2± 0.05^{fg}	0.0257± 0.02^{f}	6.33± 0.06^{i}	155.01± 0.02^{i}	280.1± 0.05^{ij}	2.716± 0.05^{j}
Dextrane $_{(standard)}$	86.4± 0.04^{f}	42.6± 0.06^{f}	132.2± 0.05^{f}	9.2± 0.05^{f}	14.7± 0.05^{f}	0.0261± 0.04^{f}	6.37± 0.01^{i}	156.56± 0.06^{i}	284.1± 0.06^{ij}	2.703± 0.01^{h}
SA $_{(Formulation)}$	81.5± 0.02^{f}	39.1± 0.04^{f}	127.8± 0.06^{f}	8.6± 0.04^{f}	12.1± 0.08^{f}	0.0238± 0.06^{f}	5.33± 0.05^{i}	150.01± 0.05^{i}	265.5 ± 0.02^{i}	2.596± 0.06^{h}
SA $_{(stand)}$	82.5± 0.01^{f}	39.6± 0.04^{f}	128.2± 0.05^{f}	8.8± 0.06^{f}	12.5± 0.06^{f}	0.0241± 0.05^{f}	5.37± 0.06^{i}	151.56± 0.06^{i}	270.1± 0.01^{i}	2.601± 0.04^{h}
PF23+ EPS	87.3± 0.01^{fg}	43.2± 0.01^{f}	134.1± 0.01^{f}	9.4± 0.02^{f}	16.2± 0.01^{fg}	0.265 ± 0.01^{fg}	6.46± 0.02^{i}	158.01± 0.04^{i}	286.3± 0.01^{ij}	2.720± 0.01^{hi}
PF23 +SA	83.1+0.02^{f}	40.2± 0.01^{f}	129.2± 0.01^{f}	9.0± 0.01^{f}	13.1± 0.02^{f}	0.251 ± 0.02^{f}	5.41± 0.02^{i}	152.61± 0.04^{i}	272.3± 0.03^{i}	2.610± 0.01^{h}
SA + EPS	96.8± 0.04^{fg}	45.1± 0.05^{fg}	138.5± 0.05^{fg}	11.1± 0.08^{fg}	17.6± 0.09^{fg}	0.0281± 0.06^{g}	7.95± 0.07^{ij}	160.51± 0.01^{ij}	290.7± 0.01^{j}	2.978± 0.03^{ij}
SA + EPS $_{(standard)}$	98.5± 0.05^{fg}	46.7± 0.07^{fg}	140.2± 0.06^{fg}	12.8± 0.04^{fg}	18.7± 0.05^{fg}	0.0287± 0.05^{g}	7.88± 0.04^{ij}	161.72± 0.04^{ij}	295.2± 0.02^{j}	2.988± 0.05^{ij}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (P = 0.05) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replicates

Chapter 5

Discussion

Soil salinity is a serious environmental problem especially in arid and semiarid areas of the world. It either occurs naturally or is human-induced. High levels of soil salinity negatively affect crop growth and productivity leading to land degradation (Allbed and Kumar 2013). When soil salinity is high, plants become even more susceptible towards the attack of phytopathogens, showing severe symptoms (Egamberdieva et al. 2014a). Combined effect of these abiotic (salinity) and biotic (phytopathogen) factors diminish plant growth yields and eventually leads to degraded lands.

World population is continuously increasing and it is estimated to reach ~8 billion by 2020. There is a real challenge to feed this huge population; an endeavor that requires agricultural productivity to be increased. Thus, obtaining high yields is the main challenge for agriculture, but the constraints associated with biotic and abiotic factors play limiting role. Different methods of chemical amelioration have been used to control these diseases and enhance productivity in saline lands. However, uncontrolled use of chemical fertilizers and fungicides has severely affected the agro-ecosystem. Increased dependence on chemicals is inevitably associated with environmental and health hazards. In this regards, PGPRs and their metabolites have been found as effective, eco-friendly and sustainable replacement to the harmful chemicals (Shaikh and Sayeed 2015).

In the present study 110 fluorescent bacteria were obtained from the rhizosphere of diverse crops grown in the semiarid region of west Kanpur and adjoining areas. The isolates were divided into three groups according to their morphological appearance viz. large fluorescent colonies with diffused margins; small, transparent, slightly fluorescent colonies and intermediate colonies with red pigment. Isolates were further characterized on the basis of physiological and biochemical characters as described in Bergey's Manual of Systematic

Bacteriology (Garrity 2005). Production of fluorescent pigment is characteristics of the so called fluorescent Pseudomonads and production of these compounds is sufficient *per se* to allocate a strain in the genus *Pseudomonas* (Garrity 2005). Fluorescent Pseudomonads are one of the most studied PGPR, however, they are very less explored in the Indo Gangetic region particularly from semi-arid regions (Yadav et al. 2014).

Amongst all the isolates, sixteen showed biochemical characteristics that differentiated them from other members of fluorescent *Pseudomonas*. All the sixteen isolates displayed similarity in morphological, physiological, carbon source utilization pattern, growth kinetics and were given the vernacular name 'fluorescent *Pseudomonas* group 'PF07-PF23'. Isolates were further screened on the basis of stress tolerance, PGP and biocontrol traits. Two potent isolates PF23 and PF17 were selected from the collection as they displayed maximum survivability upto 2000 mM and 1400 mM NaCl respectively. Different species of *Pseudomonas* viz *P. alcaligenes, P. stutzeri, P. aurantiaca* and *P. rathonis* have been reported from semi-arid soils displaying survivability upto 7% NaCl (Egamberdiyeva and Höflich 2004). Universal distribution and remarkable degrees of physiological adaptations make Pseudomonads one of the most studied bacteria (Selvakumar et al. 2015). Surendran et al. (1983) grouped bacteria on the basis of salt tolerance and further subdivided them into slightly halophilic (requiring 2-5% salt), moderately halophilic (requiring 5-20% salt) and extremely halophilic (requiring 20-32% salt). Thus, it could be accounted that PF17 and PF23 were moderate halophiles as they tolerated 8% and 12% NaCl respectively. PF23 and PF17 displayed high osmotolerance, increase in salinity upto 100 and 200 mM NaCl stimulated the growth of PF23 and PF17 respectively. Several workers reported stimulation of bacterial growth with slight increase in NaCl concentration (Hua et al. 1982; Arora et al. 2000; Arora et al. 2006). Sherman et al. (1922) showed the accelerating effect of certain salts on the growth of bacteria due to an increase in the growth rate.

These stress tolerating isolates were further identified on the basis of 16S rRNA and PCR- ITS analysis. Phylogenetic relatedness of PF23 was inferred from distance matrix based nucleotide sequence homology, which showed 99% nucleotide relatedness with *P. aeruginosa* (Gen Bank accession number AB691548.1 and KC417305.1). Based on biochemical, physiological characteristics, and nucleotide homology, isolate PF23 was identified as *P. aeruginosa* and sequence data has been submitted to NCBI Gen Bank with accession number KF598858. Isolate PF17 displayed similarity with *P. fluorescen*s on the basis of morphological, physiological, biochemical and ITS regions. Yadav et al. (2014) reported that prokaryotic phylogenetic tree provides graphical similarities among species, which is useful to derive the phylogenetic relationship amongst microorganisms. Advantage of phylogenetic network is that a strain in the network (terminal or internal node) can have multiple affinities that are incompatible with the strict branching pattern of a tree.

The virulence of *P. aeruginosa* is multifactorial, but it is determined by exoproducts such as protease and elastase, which are responsible for damaging the tissues by degrading elastin, collagen and proteoglycans (Vermelho et al. 1996). These enzymes have been also shown to degrade proteins that function in host defense (Sakata et al. 1993). Proteases play a crucial role in numerous pathological processes. Arthritis, tumor invasion, metastasis, infections and number of degenerative diseases have been linked with the involvement of one or more proteolytic enzymes (Brown 1994). Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms. Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases (Lantz and Ciborowski 1994). *P. aeruginosa* that produces proteases and elastase have been implicated in pathogenicity (Egamberdiyeva 2005). In the present study both the fluorescent Pseudomonads PF23 and PF17 were found negative for proteases and elastase activity, suggesting them to be avirulent and non-pathogenic thereby confirming there biosafety issues.

Strain PF23 and PF17 taken in the study displayed diverse PGP and biological control attributes under saline conditions. Strain PF23 showed production of EPS and SA upto 2000 mM and 500 mM NaCl respectively, whereas, PF17 showed multiple attributes including IAA production, P solubilization, Zn solubilzation, siderophore production, cyanogenic activity, pyocyanin, lytic enzyme production upto 600 mM NaCl.

Pseudomonas are prolific producers of diverse secondary metabolites working under saline conditions (Mehnaz 2014; Arora 2015; Pandya and Saraf 2015). Qualitative and quantitative results of PF17 confirmed that bacteria produced varied metabolites that were maintained as such upto 200 mM NaCl (similar to 0 mM NaCl), followed by gradual reduction in these attributes. It was monitored that upto 200 mM NaCl growth of PF17 was maintained as such (significantly similar to 0 mM NaCl). Study indicates that maintenance of growth or bacterial population is the important factor for continuing PGP and biocontrol traits under stress conditions. As salinity increases beyond 200 mM, bacterial population reduced and hence, PGP and biocontrol potential also declines gradually. Previous reports of Sandhya et al. (2010) also confirmed reduction in metabolite production with increase in salinity. Khare et al. (2011) reported *P. aeruginosa* EKi was able to produce IAA, siderophore and pyocyanin with gradual reduction of upto 76.31, 45.46, and 48.99%, respectively, as NaCl concentration increased from 0 to 500 mM. Deshwal and Kumar (2013) also accounted the effect of NaCl on the PGP activity of *P. aeruginosa*, *P. putida*, *P. cepacia* and *P. fluorescens*. Study reported that maximum metabolites (IAA, HCN, siderophores, P solubilization) were produced upto 1.25% NaCl conditions and as salinity increased above 1.25% it delayed metabolite production.

Several workers reported the role of IAA, pyocyanin, siderophores, P solubilization, HCN in growth promotion and disease suppression (Voisard et al. 1989; Perneel et al. 2007; Khare et al. 2011). However, to be effective in arid conditions metabolic machinery in the bacteria are needed to be effective even during stress conditions. The results of the study highlighted that PF17 displayed strong antagonism against *M. phaseolina* in plate assay, in tube, pot and field trails. There was gradual reduction in concentration of pyocyanin, siderophore and HCN with increase in NaCl concentration, and these metabolites might have played significant role in biocontrol. There are reports of biocontrol activity of pyocyanin against *Colletotrichum falcatum*, *Fusarium oxysporum* and *Sclerotium rolfsii* (Rane et al. 2008). Siderophores also contribute to disease suppression by conferring competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Loper and Henkels 1999). Saha et al. (2012) reviewed the role of microbial siderophores dealing with enhanced PGP and biocontrol traits. Earlier studies of Voisard et al. (1989) reported the role of HCN in disease inhibition under *in vitro* conditions. Cyanide is a secondary metabolite produced by the members of the genus *Pseudomonas,* which have unfavorable effects on the growth of soil pathogens (Prı´ncipe et al. 2007), but its production under saline conditions is not researched upon. Khare et al. (2011) reported the role of antifungal traits such as siderophores, HCN, lytic enzymes in inhibiting phytopathogens.

Cell wall of *M. phaseolina* is made up of chitin, cellulose and lipids, secretion of extracellular lytic enzymes like chitinase, β-1,3 glucanase, cellulase and lipase by PF17 worked in degrading fungal cell wall. Gamalero and Glick (2011) reviewed that some biocontrol strains produce lytic enzymes that can lyse cell wall of many pathogenic fungi. Many biocontrol Pseudomonads produces chitinase, β-1, 3 glucanase and cellulose that digest mycelium of fungal pathogens like *Fusarium* under non-saline conditions have been reviewed by Pandya and Saraf (2015) but its functioning under saline conditions is new. Phase contrast microscopy from the zone of inhibition displayed cell wall lysis and hyphae perforation thereby reconfirming the biocontrol activity of PF17 in antagonizing *M. phaseolina*.

PF17 was further taken for *in vitro* (tube study) and *in vivo* (pot) study under saline and non-saline conditions. It was observed that untreated seeds displayed poor germination and growth in comparison to the seeds treated with PF17. High salt concentration in soil causes reduction in osmotic potential, which lead to reduced water availability in root cells and make difficulties for plant to obtain both water as well as nutrients (Marschner 1995; Singh 2001). Consequently, a rapid reduction happens in growth rate, productivity along with many metabolic changes (Talei et al. 2012). Salinity plays a significant role in determining the level of nutrients like Fe, P, Zn in soil (Malvi 2011). Under salinized soil these very essential nutrients get precipitated or become unavailable to plants, thus hampering growth and production.

Treatment of seeds with PF17 brought significant enhancement in plant growth parameters under both saline and non-saline conditions. Treatment of seeds with PF17 brought 109.4% enhancement in seed yield under saline conditions in comparison to control (in pot study). PF17 synthesized growth hormone IAA that might have played significant role in germination, root length, shoot length proliferation. It is believed that approximately 80% of rhizobacteria produce IAA (Parmar and Dufresne 2011). Khare and Arora (2010) reported that production of IAA, by *Pseudomonas*, has been associated with plant growth promotion, especially root initiation and elongation, and has an indirect role in disease suppression. The biosynthesis of IAA in rhizobacteria is affected by several environmental factors especially salinity (Egamberdieva et al. 2014b). In particular, IAA production increases in conditions of higher pH, limited carbon, and higher quantities of tryptophan (Spaepen et al. 2009). Egamberdieva and Kucharova (2009) reported the role of IAA producing pseudomonads in stimulating germination, shoot, root length and dry matter of chickpea in saline soil. Thus IAA may be one of the factors responsible for growth promotion of sunflower under saline conditions.

Several workers reported high salinity decreases concentration of essential nutrients like P, Zn and Fe in both root and shoot system of sunflower seedling (Sanchez and Delgado 1996; Santos-dos et al.1999; El-Kader et al. 2006; Arora 2015). PF17 secreted bunch of PGP metabolites that displayed iron sequestration, zinc solubilization and P solubilzation thereby, increasing the mobility of Fe, Zn and P in saline soil and enhancing plant growth. Previous study of Kasotia et al. (2012) accounted the role of *Pseudomonas* in promoting soybean growth, when grown at 200 mM salt stress. Ameliorative effect of *Pseudomonas* on plant growth under saline conditions has been shown for different plant species. Inoculation of beans with *P. extremorientalis* and *P. chlororaphis* increased shoot length of bean at 5.0, 7.5 and 10.0 dS/m upto 50% (Egamberdieva et al. 2013). In earlier studies Hasnain and Sabri (1996) showed that inoculation of wheat with *Pseudomonas* stimulated plant growth under saline conditions. Several workers suggested that PGPR can stimulate plant growth by increasing P solubilization and facilitating the uptake of nutrients by the plants under saline conditions (Kloepper et al. 1989; Glick 1995; Chabot et al. 1996; Biswas et al. 2000).

PF17 also displayed production of siderophore upto 600 mM salinity. Secretion of siderophore is an important characteristic displayed by PF17 which might have played its role in plant growth promotion of sunflower. Saline soils are also deficient in iron (Neilands 1981).

Siderophores provide an advantage in the survival of both plants and bacteria because they mediate competition that results in exclusions of fungal pathogens and other microbial competitors in the rhizosphere by a reduction in the availability of iron for their survival (Wang et al. 2000). Although some siderophores are known to chelate other ions, their specificity to iron is the most consistent feature (Chincholkar et al. 2007). Several evidences indicate that siderophore production when iron is limited is responsible for the antagonism of some strains of *P. aeruginosa* against *Pythium* spp. (Antoun and Prevost 2005). Also, HCN expression and production by *Pseudomonas* was strongly dependent on iron availability (Keel et al. 1989) and may act synergistically with siderophores. Siderophores produced by rhizosphere microorganisms have been considered not only to improve rhizosphere colonization of the producer strain but to also play an important role in iron nutrition of the plant (Vansuyt et al. 2007).

Hence it could be made out that multifaceted and diverse mechanisms of PF17 are working together in promoting plant growth, protecting plant health and offering protection from phytopathogen under saline conditions. PF17 chelated iron and produced HCN. HCN expression in *Pseudomonas* is strongly dependent on iron availability and synergistically with siderophore (Keel et al. 1989; Arora et al. 2014). Production of HCN is closely linked with siderophore metabolism (Blumera and Haas 2000). Siderophore producing strains of *P. aeruginosa* releases organic acids which participate in Zn and P solubilization (Mahmod and Allah 2001). Pyocyanin is a type of siderophore produced by PF17 under saline conditions. Pyocyanin produced by *P. aeruginosa* functions as an antibiotic and plays significant role in biocontrol of phytopathogen *M. phaseolina* (Khare and Arora 2011). Extracellular lytic enzymes released by PF17 are primarily implicated for biocontrol function but in fact are more for degradation of biopolymer providing nutrients to microbes and plants and resulting in mineral cycling that is most important for sustainability.

On the other hand strain *P. aeruginosa* PF23 taken in the study though didn't displayed the production of diverse metabolites as presented by PF17, but it produced two effective key metabolites EPS and SA under saline conditions. PF23 displayed salt tolerance upto 2000 mM NaCl and displayed 1.323 g/l of EPS production (at 2000 mM NaCl). Progressive increase in salinity (from 100 mM to 2000 mM NaCl) brought significant enhancement in EPS production. Mutational study was conducted to confirm the role of EPS and SA in disease suppression and stress amelioration. It was observed that EPS defective mutant $PF23^{EPS-}$ didn't display salt tolerance above 200 mM and showed significant reduction in EPS production in comparison to wild strain (at 200 mM salinity). Thus a strong correlation could be observed between EPS production and salinity tolerance. At low salt concentration (upto 500 mM NaCl) PF23 displayed 0.901 g/l EPS that constituted mainly of glucose and galactose at its components. Whereas, further increase in salinity (up to 2000 mM NaCl) resulted in glucose, rhamnose, mannose and trehalose as major constituents of EPS. Strain PF23, its CFCS and purified EPS displayed strong biocontrol potential against *M. phaseolina* upto 500 mM NaCl, suggesting the presence of bioactive compounds in EPS (mainly glucose and galactose) that function well at low level of salinity. Several workers reported the role of sugar analogs (2-deoxy-D-glucose) in inhibiting the growth of yeasts and filamentous fungi (Barnett and Lilly 1951; Atkin et al. 1964; Krátky et al. 1975; El Ghaouth et al. 1995). Apart from it El Ghaouth et al. (1997) reported the role of glucose in inhibiting the radial growth of several pathogens and causing morphological alterations in *Rhizopus stolonifer* and *Botrytis cinerea.* Although, the inhibitory activity of EPS in antagonizing dreadful phytopathogens has been illustrated by several workers (Shankar et al. 2010; Anju et al. 2010; Orsod et al. 2012) but its role under saline conditions is little known.

Though, EPS actively participated in biological control of pathogens (upto 500 mM salinity), its role could also be related to stress amelioration. Addition of NaCl (above 500 mM) in the medium stimulated the mucoid growth (profuse spreading of the EPS) of the PF23 up to 2000 mM. Increase in EPS production with increase in salinity suggests that under stress condition energy flow of the PF23 is directed towards protective mechanism, and synthesis of EPS is opted as a defensive strategy for maintaining its survivability and ameliorating salt stress (Räsänen et al. 2004; Ashraf et al. 2006). Discharge of EPS results in aggregation/flocculation and sheath formation around the cell, which significantly improves survival of bacterial cells in saline stress (Joe and Sivakumaar 2009). Variations in the saccharide composition with increase in salinity indicate structural differences amongst the different polymers. EPS isolated during stressed conditions was composed of multiple saccharide units like rhamnose and trehalose. Role of mannose, rhamnose and trehalose as stress ameliorators has been explained by Crowe and Crowe (1992). Trehalose protects cellular enzymes by replacing water around macromolecules (Webb 1965) and also stabilizes cell membranes during stress conditions (Crowe and Crowe 1986). Under unfavorable conditions synthesis of EPS, with multiple sugars stabilizes cellular structure and offers protection from stress (Gaballa et al. 1997). Thus it may be suggested that by storing large quantities of carbon in the form of EPS during osmotic stress, PF23 cells may balance the osmotic pressure imposed by the environment and act as a stress ameliorator or osmoprotective agent.

EPS producing strain $PF23^{EPS+}$ and its mutant $PF23^{EPS-}$ were checked for SA production ability. It was found that the mutant strain was not only defective in EPS production but also displayed significant reduction in SA production under non-saline (0 mM NaCl) conditions, followed by complete loss with increase in salinity. However, wild strain PF23^{EPS+} displayed SA production up to 500 mM NaCl. Hence a relationship could be established in between SA and EPS production. Signaling crosstalk in *Agrobacterium tumefaciens,* where SA promotes EPS synthesis has been demonstrated in the past. Regulation of *exo* genes involved in EPS synthesis and carbon metabolism by SA has been reported earlier in diverse bacterial strains (Leigh et al. 1985; Yuan et al. 2008). Several workers also reported the role of EPS and SA as signal molecules (Kunkel and Brooks 2002; Wang et al. 2007; Lamothe et al. 2012). AHL synthase gene *swrI* produces quorum sensing molecule, C_6HSL (N-hexanoyl L-homo-serine lactone), that induces SA production in *Serratia liquefaciens* (Schuhegger et al. 2006). Whereas, *Pantoea ananatis* produces AHL synthase gene *eanI* that produces same signal molecule, C_6HSL, that regulates EPS synthesis (Morohoshi et al. 2007). Mutation in AHL synthase gene inhibits the synthesis of metabolites thereby suggesting link in between the two (Rad et al. 2008; Morohoshi et al. 2007). During iron limitation, enhanced gene expression of EPS and SA was observed in *Sinorhizobium meliloti* and *P. fluorescens* (Chao et al. 2005). Based on earlier studies and the present one it might be concluded that SA and EPS have strong connection and may have overlapping regulatory mechanisms hinting at molecular control in between both the metabolites.

The production of SA by PF23^{EPS+} was detected up to 500 mM NaCl, as monitored both by qualitative and quantitative analysis, and was further authenticated by HPLC chromatogram. Toiu et al. (2011) reported R_T of SA at 4.8 min as obtained on HPLC chromatogram. Shanmugam and Narayanasamy (2008) reported that Rf value and $R_{T\ of}$ SA purified from *Bacillus licheniformis* was found to be 0.60 and 5.24 min, respectively, which is quite similar to the results of this study. PF23^{EPS+} displayed maximum SA production and biocontrol against *M.*

phaseolina under control conditions that was significantly similar to the results obtained at 100 mM NaCl. However, subsequent increase in salt concentrations brought reduction in both SA production and biocontrol potential. As the concentration of SA decreased, inhibitory activity against pathogen also got reduced. The study thus reports the role of SA in antagonism under saline conditions. The mutant strain neither displayed SA production nor antagonism against *M. phaseolina*, under saline conditions, which further confirmed the role of SA in biological control. SEM analysis also confirmed the role SA in antagonism as it showed deformities in fungal mycelium and hyphae perforations. The role of SA producing stress tolerating strain of *B. licheniformis* in biological control of *M. phaseolina, Bipolaris oryzae, Pyricularia oryzae, Curvularia lunata, Fusarium oxysporum* and *Alternaria alternate* have been reported by Shanmugam and Narayanasamy (2008). Nehal and Mougy (2004) reported the inhibitory activity of purified SA on development and sporulation of *Fusarium solani* and *Sclerotium rolfsii.* They also discussed the role of SA in antagonizing bacterial pathogens like *Bacillus polymyxa, Erwinia caratovora* and *Pseudomonas solanacearum.* Though several workers discussed the role of SA in inhibiting pathogens, but its antagonistic role under saline conditions and with respect to EPS producing microbes is novel and still needs to be worked upon. *In vitro* (biocontrol activity) studies confirmed the active role of EPS and SA in biological control.

P. aeruginonsa PF23 was further subjected to *in vitro* (tube) and pot study with sunflower. *In vitro* tube study and pot study showed that biopriming of seeds with PF23^{EPS+} displayed significant increase in germination in comparison to unprimed seeds under non-saline and saline conditions. Biopriming with PF23 brought increment in plant growth parameters and yield, whereas EPS defective strain did not enhanced germination, plant growth parameters and yield under saline conditions. However, under non-stress conditions, PF23$^{EPS\neg}$ brought increase in shoot length and seed yield in comparison to *M. phaseolina* infested seeds, suggesting the role of other PGP metabolites like siderophore and IAA in growth promotion and yield enhancement as PF23^{EPS-} was positive for these PGP characteristics only under non-saline conditions. Mutant PF23$^{EPS¬}$ under stressed conditions was ineffective in enhancing plant growth parameters suggesting absence of PGP activity under saline stress conditions, confirming the involvement of EPS and SA in plant growth promotion under saline stress.

Results of both tube and pot study showed the effectiveness of PF23 and PF17 in enhancing sunflower growth under saline environment. However, under certain cases, the results obtained in the laboratory could not be reproduced in the field (Zhender et al. 1999; Smyth et al. 2011; Arora 2014; Arora 2015). This might be due to the low quality of the inocula and/or the inability of the bacteria to compete with the indigenous population under adverse environmental conditions (Brockwell and Bottomley 1995; Catroux et al. 2001). Great variations in the plant response to PGPR in laboratory and field assays demonstrate that the full potential of rhizobacteria to promote plant growth should be more extensively investigated. It is necessary to develop efficient inocula that can perform better under field conditions (Ahmad et al. 2008). Hence, there is an urgent need for developing multifaceted bioformulation that can enhance plant growth, display biocontrol potential and ameliorate stress. Formulations that transfer the growth promoting activities of an isolate from laboratory to field would have a major impact in agriculture.

In present study six carriers were screened on the basis of WHC, IMC, pH and shelf life. Amongst all the selected carriers talc proved best in supporting bacterial population both under stress and non-stress conditions. The carriers taken in the study were sterile, as this form maintains the high population density in the formulation. The use of sterile carriers significantly reduces the threat to the quality of bio-inoculants arising from the presence of contaminants and other autochthonous microorganisms (Stephens and Rask 2000). There are reports that carriers possessing high WHC and near-neutral pH can support large bacterial populations (Roughley and Vincent 1967; Arora et al. 2008). Kadouri et al. (2005) reported that bacterial cells taken for developing formulation should maintain their survivability under stressful conditions for displaying effective performance in field. It was observed that after talc, coriander husk was the most efficient carrier in supporting bacterial population both under stress and non-stress conditions followed by sawdust (in both the strains PF17 and PF23). Previous reports of Arora et al. (2014) also reported the survivability of *Pseudomonas* and *Rhizobium* isolates in coriander husk and sawdust upto five weeks suggesting their efficacy as potent carrier.

Diverse types of cell based and metabolite based bioformulations were developed taking talc. These talc based formulations not only enhanced plant growth parameters of sunflower but also suppressed charcoal rot disease incidence. Previous report of Ardakani et al. (2010) reported, the use of mineral carriers (such as talc) together with antagonistic bacteria have been reported effective in controlling disease in plants and enhancing plant growth. It was found that formulation developed from stressed cells of PF17 and PF23 were far better in maintaining population density in comparison to that developed from unstressed cells. De Castro et al. (2000) reported that lowered water content increases the long term stability of the dried bacteria during storage. Freeze drying causes extraction of water that leads to the accumulation of salts resulting in increased shelf life (Khare and Arora 2011).

Different formulations were developed from cells and metabolites of PF23 and PF17 by embellishing talc powder. Cell and supernatant based formulations developed from PF17 and PF23 displayed significant enhancement in plant growth parameters of sunflower. Formulations developed from the combination of metabolites (SA and EPS) and blend of PGPR cells along with metabolites also prove to be effective in enhancing sunflower production. However, the best results were obtained when formulation was developed from the combination of purified metabolites and cocktail of PGPR cells along with metabolites.

Cell based formulation developed from stressed cells of PF17 and PF23 enhanced seed yield by 74.2% and 133% in salinized soil and suppressed charcoal rot incidence in sunflower. Karthikeyan *et al*. (2012) reported that talc based formulation developed from cultures of ***Bacillus subtilis,*** *P. fluorescence, Gliocladium virens* and *Trichoderma* spp. could significantly reduce leaf blight incidence and increased growth of onion plant both in glasshouse and field trials. Anand *et al*. (2010) also accounted the use of talc-based formulation of *P. fluorescence* in suppressing growth of fruit rot caused by *Colletrotichum capsicii* and powdery mildew caused by *Leveillula taurica*. Jorjani et al. (2012) also pointed out that talc-based formulation of *Pseudomonas* strains (Talc-B1 and Talc-B2) was effective against sugar beet disease. These data show that cell based formulations developed from talc were performing well, but, most of their studies are under non-saline field conditions, However, there are very few reports of successful application of cell based formulations in salinized or degraded soils (Bashan et al. 2014).

However there are certain constraints associated with the application of cell based formulations (Arora et al. 2010; Bashan et al. 2014; Mishra et al. 2015). Shaikh and Sayeed (2015) reported that cell based formulation provides inconsistent field performance, and do not perform well in the field conditions. These failures have raised concerns about the perspective of the great practical potential offered by microbial releases into soils. A key factor involved in the failure is the rapid decline of the size of the population of active cells. The active cells decline to a level at which the formulation becomes ineffective when introduced into the soil and the desired objective is not achieved. Hence in order to overcome these constraints remedial measures should be taken and more focus should be given on metabolites based formulations or (Lughtenberg 2015; Nadeem et al. 2015; Morel et al. 2015).

In the present study CFCS and metabolite based formulations were also developed from PF17 and PF23 and their delivery in the field gave successful results. Talc based formulation developed from CFCS of PF17 and PF23 showed even better enhancement in growth parameters and suppressed disease incidence in comparison to cell based formulation (both under stressed or non-stressed state). Reason for such increase in growth attributes and disease management might be due to the secretion of diverse extracellular metabolites (P, Zn, IAA , siderophore and lytic enzymes) by PF17 and two interdependent metabolites (EPS along with SA) by PF23. These metabolites were even maintained under saline conditions and hence participated actively in growth promotion and disease management. Bioformulation developed from *P. fluorescence* was effective in enhancing PGP attributes of sugar beet, as its supernatant contained diverse metabolites such as siderophore, hydrolytic enzymes, phytohormones and/or other volatile extra-cellular metabolites (Sutruedee et al. 2013).

Lughtenberg (2015) reported that lipopolysaccharides (LPS), have been clamied to be responsible for the antimicrobial effects exerted by *B. subtilus*. However, it is very likely that the concentrations of these aniifungal metabolites within plant rhizosphere do not reach sufficeint level for antibiosis. Hence, to circumvent this problem bioformualtion containing both *Bacillus* cells and concentrated culture supernatant containing antimicrobial metabolites should be developed. An improved formulation rely on cocktails of metabolites or plant growth regulators (PGR) along with bacterial cells (Lughtenberg 2015). To improve the effectiveness of inoculum and enhance reproducibility, Frankenberger and his co-workers (1987) developed the idea of precursor-inoculum interaction based upon the hypothesis that inoculation of PGPR in the presence of specific physiological precursor of a plant growth regulator (PGR) or metabolite is often more effective in promoting plant growth than inoculation alone (Naveed et al. 2015). In the present study formualtion designed by combining the cells along with metabolites (PF23 cells + EPS and PF23 cells + PF17) gave significant enhancemnt in seed yeild in comparision to cell based formulation and individual metabolite based formulations, therby suggesting the importance of biomolecules based formualtions.

Frankenberger and Poth (1987) conducted greenhouse experiments with ectomycorrhizae, *Pisolithus tinctorius* and L- tryptophan on the growth of *Douglas fir*. Results revealed that growth of *D. fir* was stimulated by *P. tinctorius* inoculation in supplementation with lower concentrations of L- tryptophan most probably due to interaction of soil indigenous microbiota with metabolite L- tryptophan. Hussain et al. (1995) observed that *Rhizobium* inoculation alone increased the grain yield of lentil by 21% over uninoculated control; however, when combined with L- tryptophan, the *Rhizobium* inoculation further increased the lentil yield by 31% over control.

Further, it was observed that metabolite based formulation developed from PF23 gave significant results in enhancing growth of sunflower and suppressing disease incidence in comparison to individual treatments of EPS and SA or blend of cells along with metabolites. Whereas, effective results were obtained when CFCS of stressed PF23 and PF17 were applied.

Under saline conditions there was significant reduction in all the plant growth attributes and yield in case of unprimed seeds. Reason for reduction in growth parameters might be the alteration in physiology of sunflower plant induced by salt stress that reduced nutritional and water uptake (Arora et al. 2012). The decline of seed germination might be due to decrease of water influx, which reduced seed humectation required for metabolic reaction involved in germination processes (El Midaoui et al. 2001). Priming of seeds with the combination of purified SA and EPS brought increment in germination by 56% in comparison to unprimed seeds. Role of SA and EPS has been elucidated in seed germination and seedling growth, serving as a kernel priming agent (Nazar et al. 2011; Tewari and Arora 2014a). The amalgamated combination was significantly effective in increasing plant growth parameters and suppressing disease incidence. Pre-sowing application of seeds with combination reduced negative impact of salinity and enhanced plant growth and seed yield under saline conditions and in presence of pathogen. SA has been previously reported as a plant growth regulator with a significant role in recuperating of physiological processes in plants (Sakhabutdinova et al. 2003). SA can elongate the plant cells and increases cell division and therefore, results in improving plant length, leaf area, stem width and chlorophyll content. Similar results of growth improvement by SA were also reported by Raheleh et al. (2013) in maize under saline conditions. SA is also known to induce antioxidative response and membrane protection thereby preparing the plant against the impact of salt stress.

Application of EPS increased RAS/RT ratio, upsurges adhesion of soil particles, intensifies soil aggregation, enhances soil texture, increases water holding capacity of soil, reduces water loss during stress conditions and protects plant from pathogenic invaders (Tewari and Arora 2014b). Increased RAS/RT upsurges adhesion of soil particles, intensify soil aggregation, enhances soil texture, increase water holding capacity of soil and reduce water loss during stress conditions and protect them from pathogenic invaders (Roberson and Firestone 1992). Increased RAS on PF23 application showed that hydrophilization of soil leads to improved supply of nutrients that is responsible for plant growth promotion and disease suppression (Alami et al. 2000). Microbial EPS and SA released by PF23 also assist in minimizing the effect of salt stress by functioning as ameliorating agents. Hence, multiple roles of EPS were illustrated by the study proved that at lower concentrations (upto 0.901 g/l or 90 μg/ml EPS) EPS can function as a biocontrol metabolite that possesses antagonistic activity against dreadful phytopathogen *M. phaseolina*. As the concentration of EPS increased with progressive increase in salinity, it started to behave more as an osmoprotective agent (above 0.901 g/l EPS) and thus protected PF23 and sunflower from the plethora of osmotic stresses. Finally, when this EPS producing PF23 was introduced in salinized soil it served as a biopriming agent. The plant hormone SA and EPS are also reported to regulate signaling networks, involved in inducing plant immunity (Jones and Dangl 2006; Zipfel 2009). El Oirdi et al. (2011) reported EPS induced accumulation of SA conferring resistance against phytopathogen *Pseudomonas syringae* in tomato.

Thus amalgamation of SA and EPS might have worked synergistically to protect the plant from attack of phytopathogens, reduce the incidence of disease and enhance growth and productivity under salinized conditions. Kaya et al. (2002) reported that SA is involved in protecting plants under abiotic and biotic stresses including salinity. Horvath et al. (2007) reported that treatment of seed with SA protects plants against abiotic stresses. SA a metabolite reported commonly to be produced by pseudomonads (Meyer and Hofte 1997) has a role in induced resistance (Ran et al. 2005). EPS is also induced under abiotic stresses such as salinity and known to protect the bacterial cells under these conditions (Upadhyay et al. 2011). Both these metabolites being induced under saline conditions may have some linkage or regulation. EPS and SA (as pure metabolites or cells producing them) can thus be used to control the phytopathogens and enhance productivity under saline conditions. For sustainability in the agriculture sector, blend of microbes and microbial products can be used for cumulative effects (Lughtenberg 2015; Nadeem et al. 2015). Combination of metabolites and PGPR cells can also prove to be effective for development of future formulations as prove by the present study.

Use of chemicals and resistance shown by phytopathogens has resulted in loss in soil fertility, yield and problems such as salinity and soil pollution. The future is only for the eco-friendly measures which not only lead to sustainable and pollution free environment but also development of a market sound product instilling confidence amongst the end users. The amalgamation of EPS along with SA or metabolites and cells brought significant enhancement in sunflower growth and production in saline habitats even in presence of deadly phytopathogen, *M. phaseolina*. The amalgamation congaing multifaceted activities of stress amelioration, biological control and growth promotion was utilized for developing bioformulations. Development of such a versatile bio-formulation might remove constraints associated with the inconsistent performance of presently utilizing perilous chemical formulations. The metabolites of fluorescent Pseudomonad strains can express beneficial functions both (biocontrol and PGP) in a reliable fashion under diverse soil conditions.

The major outcome of the present study is the development of a novel bioformulation derived from cells and metabolite of Pseudomonads displaying multifaceted roles. Scope and application of the developed bioformulation technology holds wide array of spectrum and will be effective under different soil type conditions and should be effectively with broad varieties of crop. Hence such beneficial PGPRs and their metabolites can be potential biotechnological tools for sustainable agriculture. However further studies are needed to identify conditions that can contribute for the optimization of metabolites and also the combinations for low input and cheap technology development that is reliable and easy to develop and apply.

Chapter 6

Conclusion

Use of chemicals and resistance shown by phytopathogens has resulted in loss in soil fertility, yield and problems such as salinity and soil pollution. The future is only for the eco-friendly measures which not only lead to sustainable and pollution free environment but also development of a market sound product instilling confidence amongst the end users. The bioformulations developed from the amalgamation of metabolite / stressed cell / or unification of cell and metabolite brought significant enhancement in sunflower growth and production in saline habitats even in presence of deadly phytopathogen, *M. phaseolina*. Development of such a versatile bio-formulation will remove constraints associated with the inconsistent performance of presently utilizing perilous chemical formulations. The metabolites and cell based formulations of fluorescent Pseudomonads can express beneficial functions of both (biocontrol and PGP) in a reliable fashion under diverse soil conditions.

Chapter 7

Summary

Sunflower is one of the fastest growing oilseed crop grown in India. Use of sunflower oil is in great demand as it trims down the incidence of cancer, hypertension, and the cholesterol in human beings. However, nowadays sunflower production is affected badly by several biotic and abiotic stresses. Amongst abiotic factors salinity is the main constraint that limits sunflower yield, whereas in biotic factors *M. phaseolina* is the phytopathogen which needs to be tackled in an ecofriendly and sustainable manner. *M. phaseolina* is fungal opportunist that likes to take advantage of stressed plant particularly in salinized regions and causes significant reduction in yields. Continuous use of chemicals in agriculture has resulted in degradation of soil fertility, formation of barren lands, disruption of soil ecology, development of resistant pathogens and ill effects on human health. Hence, there is an emergent need to develop biopreparations which can enhance plant growth, control phytopathogens, are effective even under stress conditions and above all are ecofriendly and hence lead to sustainability. Microbe-based formulations that can suppress the growth of phytopathogens and ameliorate the effect of abiotic stresses such as salinity are real alternatives to hazardous chemicals.

In the present study, 110 fluorescent Pseudomonads were isolated from Kanpur (Uttar Pradesh) and adjoining areas. Isolates were characterized on the basis of morphological, physiological, biochemical and molecular basis. The vernacular name ‘fluorescent *Pseudomonas* group PF07-PF23 was coined for a group of 17 isolates from the rhizosphere of sunflower having characteristics that differentiate them from other members of fluorescent *Pseudomonas* spp. The isolates were monitored for stress tolerance capacity, plant growth promoting traits and biocontrol potential against *M. phaseolina*. On the basis of these attributes, two potent isolates, PF17 and PF23, were selected as they displayed maximum salt, temperature and pH tolerance. In nature all the stress conditions occur simultaneously hence multi - stress tolerance assay was conducted to check the survivability of isolates. Isolates displayed survivability when multiple shocks (of salinity, temperature and pH) were given simultaneously.

Selected isolate PF23 was subjected to 16S rRNA gene sequence analysis, and was found to be *P. aeruginosa*. The 16S - 23S ITS region from PF17 gave a single amplicon of the size of 560 bp, which confirmed that the isolate was *P. fluorescence.* Protease, elastase and gelatinase activities of both the strains were checked so as to determine the pathogenicity. These initial level tests confirmed that both the strains were non-pathogenic, avirulent and hence safe to use. However, clinical trials will also be performed to completely ensure the safety before using these strains at large scale.

Strains were monitored for their plant growth promoting attributes and biocontrol potential against *M. phaseolina* under saline stress conditions. PF17 displayed production of diverse metabolites including P solubilization, Zn solubilzation, IAA production, siderophore, pyocyanin, HCN, chitinase and β-1,3 glucanase activity under saline conditions up to 600 mM NaCl. Which might have contributed in antagonizing *M. phaseolina* even, under saline conditions.

Results of plate assay clearly showed inhibition of *M. phaseolina* by PF17 up to 600 mM NaCl. *In planta* (tube) and *in vivo* (pot) studies were conducted with sunflower, under saline (125 mM NaCl) and non-saline (0 mM NaCl) conditions, both in presence and absence of phytopathogen *M. phaseolina*. Treatment of seeds with PF17 showed significant enhancement in plant growth parameters and suppression of charcoal rot disease incidence even under saline conditions. PGP activities such as P solubilization, Zn solubilization, IAA and siderophore production displayed by PF17 even under high salinity might have participated in enhancing growth attributes of sunflower under saline conditions. Whereas, secretion of inhibitory metabolites such as HCN, pyocyanin, lytic enzymes might have helped in antagonizing phytopathogen in soil infested with it.

On the other hand *P. aeruginosa* PF23 showed production of EPS and SA up to very high salt concentrations. PF23 also displayed antagonism against *M. phaseolina* upto 500 mM NaCl in dual plate assay indicating the role of EPS and SA in controlling the pathogen. Purified EPS and SA from PF23 also showed inhibitory spectrum against *M. phaseolina* upto 500 mM NaCl. Phase contrast microscopy and SEM analysis from the zone of inhibition after treating with EPS and SA displayed perforation, curling, deformities and lysis of hyphae thereby confirming the role of EPS and SA in antagonism.

Analysis of EPS constituents by TLC revealed differences in the sugar components under varied salt concentrations. Under normal conditions (0 mM NaCl) glucose (Rf 0.42) was present as the major saccharide unit in the EPS hydrosylate, whereas EPS obtained under salt stress was composed of glucose (Rf 0.42), galactose (Rf 0.37), rhamnose (Rf 0.74), mannose (Rf 0.46), and trehalose (Rf 0.32). The FTIR spectrum of EPS produced by PF23 under different salt concentrations was analyzed and absorption bands gave typical polymeric structure of the carbohydrate containing hydroxyl group, methylene group, enol and amide group. The SEM of dried EPS obtained at 0 mM NaCl appeared to be smooth, glittering, spherical or ovoid exhibiting compact structure. However, photomicrographs of EPS obtained from 500 mM NaCl conditions appear polyhedral in shape with wrinkled surface. Multiple roles of EPS were illustrated by the study proved that at lower salt concentrations

EPS functioned as a biocontrol metabolite, but as the concentration of NaCl increased it started to behave more as an osmoprotant and when this EPS producing PF23 was pelleted on seeds and introduced in salinized soil it served as a biopriming agent.

Mutational study was conducted to further confirm the role of EPS in disease suppression and stress amelioration. It was observed that EPS defective mutant $PF23^{EPS-}$ didn't display salt tolerance above 100 mM and showed significant reduction in EPS production in comparison to wild strain. Mutant was unable to maintain its growth under saline conditions and even it lost it antagonizing property above 100 mM. Thus a strong correlation could be observed between EPS production, salinity tolerance and biocontrol.

EPS producing strain $PF23^{EPS+}$ and its mutant $PF23^{EPS-}$ were checked for SA production ability. Strain $PF23^{EPS+}$ displayed SA production up to 500 mM NaCl whereas its defective mutant lost SA production above 100 mM salt concentration. HPLC analysis revealed single peak up to 500 mM NaCl concentration for $PF23^{EPS+}$ at different salinity levels that resembled to standard SA in respect to its retention time (4.502 min) however, there, was no peak recorded in case of mutant under saline conditions. It was found that the mutant strain was not only defective in EPS production but also displayed significant reduction in SA synthesis under non-saline (0 mM NaCl) conditions, followed by complete loss with increase in salinity, hence suggesting overlapping roles between these two metabolites (EPS and SA). Treatment of sunflower seeds with PF23 cells, purified SA and EPS (alone) or in combination with bacterial cells brought significant enhancement in growth attributes of under saline conditions in tube and pot assays.

Both the strains were used to develop carrier based bioformulations. Amongst six carriers taken in the study (corn husk, coriander husk, coconut husk, charcoal, saw dust and talc), talc proved to be the best carrier in supporting the population of both the strains hence, talc based formulations were developed for field trials. Formulations were developed from normal cells (both PF17 and PF23), stressed cells (both PF17 and PF23), cell free culture supernatant (PF17 and PF23), metabolites (PF23), combination of metabolites (EPS + SA) and metabolites plus cells (PF23 + EPS or PF23 + SA).

It was observed that formulations developed from stressed cells and CFCS of PF17 and PF23 were effective in enhancing seed yield of sunflower under saline soil infested with *M. phaseolina*. However, formulation developed from metabolites (of PF23) or from cell plus metabolites proved to be best and were significantly similar in enhancing growth attributes of sunflower crop under saline conditions. Cell plus metabolites based formulation developed from EPS + PF23 and SA + PF23 enhanced seed yield by 195% and 180% respectively in comparison to untreated seeds. Combination of metabolites SA + EPS enhanced seed yield by 199.6%. The study, suggest that EPS and SA (as pure metabolites or in combination with bacterial cells) can thus be used to control the growth of phytopathogens and to enhance productivity of sunflower crop in salinized soils. The significant finding of the study encourages the use of bioformulations developed by blending PGP bacterial cells along with their metabolites.

Findings also suggest the use of such novel bioformulation (often optimization studies) for enhancing yield of such an important oilseed crop, sunflower, even under arid/semi-arid saline conditions. This ecofriendly biological product can also be used for reclamation of saline arid soils for enhanced productivity and food security.

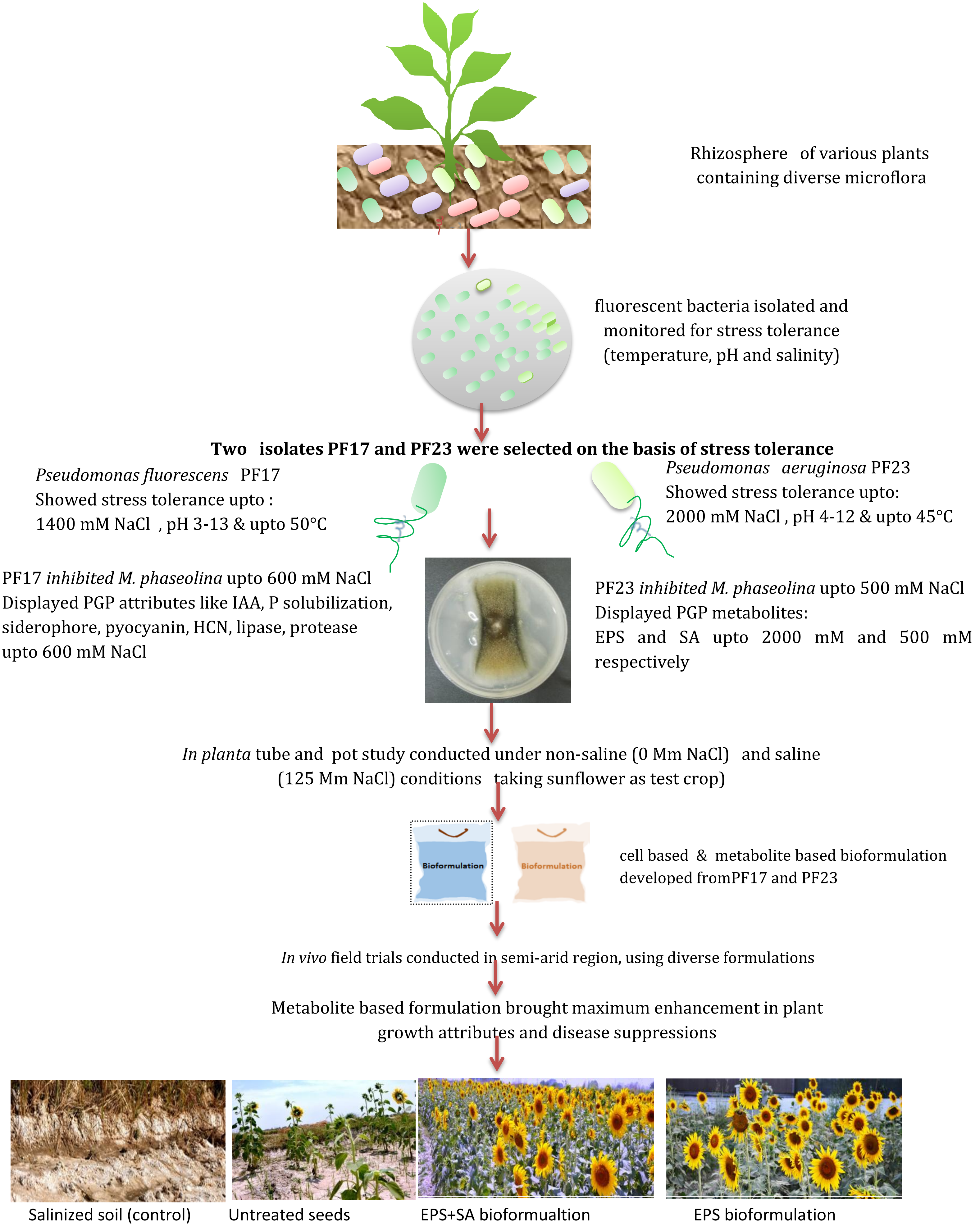


Figure 30: Summary of the work done

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1. **Tewari S** and Arora NK (2014a) Multifunctional exopolysacccharides from *Pseudomonas aeruginosa* PF23 involved in plant growth stimulation, biocontrol and stress amelioration in sunflower under stress conditions. Current Microbiology 69(4): 484-494.

2. **Tewari S** and Arora NK (2014b) Talc based EPS formulation enhancing growth and production of *Hellianthus annuus* under saline conditions. Cellular and Molecular Biology 60 (5): 71-81.

3. Arora NK, **Tewari S** and Singh R (2014) Comparative study of different Carriers inoculated with nodule forming and free living plant growth promoting bacteria suitable for sustainable agriculture. J Pharm Chem Biol Sci 2(2):143-149

4. **Tewari S** and Arora NK (2015) Plant growth promoting fluorescent Pseudomonads enchancing growth of sunflower crop. Int J Sci Tech So 1(1): 51-52.

5. Maheshwari DK, Dubey RC, Aeron A, Kumar B, Kumar S, **Tewari S** and Arora NK (**2012)** Integrated approach for disease management and growth enhancement of *Sesamum indicum* L. utilizing *Azotobacter chroococcum* TRA2 and chemical fertilizer. World J Microbiol Biotechnol 28(10): 3015-24.

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1. **Tewari S** and Arora NK (2014) Plant growth promoting rhizobacteria for ameliorating abiotic stresses triggered due to climatic variability. Climate Change Environ Sustain 1 (2) 95-103.

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Curr Microbiol
DOI 10.1007/s00284-014-0612-x

Multifunctional Exopolysaccharides from *Pseudomonas aeruginosa* PF23 Involved in Plant Growth Stimulation, Biocontrol and Stress Amelioration in Sunflower Under Saline Conditions

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Received: 8 January 2014 / Accepted: 28 March 2014

Abstract Isolate PF23 selected from among 110 fluorescent pseudomonads, identified as *Pseudomonas aeruginosa,* displayed salinity tolerance and exopolysaccharides (EPS) production up to 2,000 mM NaCl concentration. EPS-defective mutant PF23^{EPS-} of the isolate showed 86 % reduction in EPS production in comparison with wild strain. Defect in EPS production brought loss in salt tolerance capability. Purified EPS obtained from PF23 displayed multiple roles. At low concentration EPS functioned as biocontrol agent, at high concentration EPS behaved as osmoprotective or stress ameliorating metabolite and when introduced in saline soil, served as a plant growth promotor along with seed biopriming agent. Both *in planta* and in vivo studies were performed taking sunflower as a test crop and it was observed that PF23 showed plant growth promotion and significant biocontrol potential against dreadful phytopathogen *Macrophomina phaseolina* (under saline conditions). The mutant PF23^{EPS-} was ineffective under saline conditions both in growth enhancement as well as in disease suppression. The study reports a potent strain, *Pseudomonas aeruginosa* PF23, capable of enhancing production of sunflower crop in semiarid regions and minimizing the incidence of charcoal rot disease in sunflower.

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Introduction

Agriculture is considered to be one of the most vulnerable sectors, often exposed to a plethora of stress conditions. Currently, more than 800 million hectares of land throughout the world are affected by the level of salinity stress that substantially reduces crop productivity [5, 42]. Sunflower is one of the fastest growing oilseed crop grown in India. India majorly exports sunflower cake, which increased from 5,511.33 tons (triennium ending 2002) to 18,440 tons during 2009 [59]. International demand for sunflower seeds and oil is continuously increasing with the growing health consciousness and increasing knowledge on the dietary and health benefits. Sunflower is considered one of the major oil producing crops in the world due to its high quality oil, high protein content and moderate production requirements [53]. Sunflower oil is commonly used in food as frying oil, and in cosmetic formulations as an emollient, it is popular as healthy cooking oil due to its health benefits while the meal is used in animal feed industry. Use of sunflower oil is in great demand as it trims down the incidence of cancer, hypertension, and the cholesterol in human beings [49]. However, nowadays sunflower production is affected badly by several biotic and abiotic stresses and it has been estimated that among several abiotic stressors, salinity is the major constraint that limits sunflower yield, growth, and productivity. More than 60 % loss in the production of sunflower is due to salinization [29]. Unfortunately apart from soil salinity, sunflower is also attacked by variety of fungal pathogens which affect its yield and oil quality. *Macrophomina phaseolina* is a fungal opportunist that likes to take advantage of stressed sunflower plant which is cultivated in salinized regions and causes up to 70 % reduction in its oil production [26, 56]. *M. phaseolina* has a wide host range and is responsible for

Published online: 01 June 2014

causing losses on more than 500 cultivated and wild plant species [29]. As agricultural production intensified over the past few decades, producers became dependent on agrochemicals for crop protection. Continuous use of chemicals subverts the soil ecology, disrupt environment, degrade soil fertility, and consequently show harmful effects on human health along with contaminating ground water and thus creating environmental hazards [6, 28, 35]. Therefore, there is an emergent need to develop biopreparations which are ecofriendly and lead to sustainability. Microbe-based formulations that are able to suppress the growth of phytopathogens are real alternatives to hazardous chemicals. These formulations can also be used to enhance yields under environmental stress conditions. Soil-borne fluorescent pseudomonads have received particular attention as plant growth-promoting rhizobacteria (PGPR) throughout the globe because of their catabolic versatility, excellent root colonizing ability, and capacity to produce a wide range of metabolites that favor the plant to with stand under varied biotic and abiotic stresses [31]. Members of this bacterial group are versatile and able to adapt and colonize a wide variety of ecological environments throughout the world. Extracellular polysaccharides (EPS) produced by microorganisms are instrumental in imparting stress tolerance to bacterial cells [40] but relatively little attention has been paid on their utility and role under saline conditions [1].

In the present study, fluorescent pseudomonads were isolated from the rhizosphere of sunflower plants growing in saline soil. The isolates were checked for production of plant growth promotory metabolites under saline conditions. The isolates were also tested for biocontrol potential against *M. phaseolina* causing charcoal rot disease in sunflower, under saline stress condition.

Materials and Methods

Microorganisms and Growth Conditions

One hundred and ten fluorescent pseudomonad isolates from the rhizosphere of different plants from Kanpur and adjoining areas (20°38′E and 80°21′N; temperature maximum 48 °C and minimum 1 °C), Uttar Pradesh (northern India), were screened on the basis of salinity tolerance and EPS production. Isolates were tested for morphological, physiological, and biochemical characters according to Bergey's Manual of Systematic Bacteriology [22]. Among all, isolate PF23 was selected as it produced highest amount of EPS on exposure to salt stress. Isolate PF23 was further identified by analysis of 1.5 kb 16S rRNA sequences. 16S gene sequence was queried for similarities with BLAST [2] and with the Ribosomal Database Project (RDP) [30, 36]. The phylogenetic tree was constructed using Neighbor Joining method [46].

Phytopathogenic strain of *M. phaseolina* ARIFCC257 was procured from Mycology and Plant Pathology Group, Division of Plant Sciences, Agharkar Research Institute, Pune. The strain was grown and maintained on Potato dextrose agar (PDA) (Hi Media, Mumbai) at 28 and 4 °C, respectively.

Plant Growth Promoting Attributes

PF23 was checked for plant growth promotory (PGP) attributes including phosphate solubilization, indole acetic acid (IAA) production, HCN production, siderophore production, chitinase and β-1-3 glucanase activity, nitrogen fixation, and EPS production abilities under saline and non-saline conditions. Phosphate solubilizing activity of PF23 was tested by spot inoculation on Pikovskaya's medium [54]. IAA production was detected in culture filtrate using salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) [23]. HCN production was checked by observing the change in color of the filter paper impregnated with 0.5 % picric acid in 1 % Na_2CO_3 [37], whereas siderophore production was determined on Chrome-Azurol S medium according to Schwyn and Neilands [51]. Extracellular chitinase activity was determined by spot inoculation on solid chitin minimal medium (CMM), whereas β-1,3 glucanase enzyme was assayed according to Dunne et al. [14], nitrogen fixation was checked by acetlyene reduction assay [28] and EPS production was monitored by chilled ethanol precipitation method [47, 55].

Chemical Mutagenesis for Developing EPS-Defective Mutant

A loopful of PF23 cells were inoculated into 10 ml of Davis Minimal Media (DMM) broth [32] and incubated at 28 °C up to log phase. Subsequently, 100 μg ml^{-1} 5-bromouracil was added and further incubated for 02 h. Cells were centrifuged, washed thrice with sterile water, and resuspended in 10 ml of DMM broth, and mutants were fixed by overnight incubation at 28 °C. The mutant bank was stored in 25 % glycerol at −40 °C [30]. The clones of mutant bank were screened for EPS production according to Sandhya et al. [47]. Mutagenic procedure worked effectively as EPS-defective mutant, $PF23^{EPS-}$ was repeatedly checked on DMM media for stability. The EPS-defective mutants were further screened for salinity tolerance assay. The wild isolate PF23 served as a control in the mutant screening test. Maximum EPS-defective mutant was marked as $PF23^{EPS-}$.

Salinity Stress Assay

The log phase culture (OD610 = 0.1) of PF23 and $PF23^{EPS-}$ were seeded in DMM broth, amended with different concentrations of NaCl (0 to 2,200 mM) and incubated at 28 °C. Optical density was measured at 610 nm by spectrophotometer (GENESYS™6, Model, 335908-02) up to stationary phase (120 rpm). The experiment was conducted in five replicates.

Effect of Saline Stress on EPS Production

To determine the effect of saline stress (0–2,000 mM) conditions on EPS content, selected strain PF23 and its EPS-defective mutant $PF23^{EPS-}$ were grown in DMM broth, with gradients of NaCl concentrations (0–2,000 mM) for seven days. Cells were harvested by centrifugation for 10 min at 11,000×*g*. The supernatant was filtered through 0.45-µm nitrocellulose membrane; two volumes of cold ethanol were added to culture supernatants and stored overnight at 4 °C. Precipitated material was collected by centrifugation (20 min at 2,500×*g*) suspended in demineralized water, and mixed with 2 volumes of cold ethanol. Samples were centrifuged (2,500×*g*) and the pellets were dried at 100 °C and weighed. The amount of EPS was expressed as polymer dry mass and expressed in g/l [19, 20, 48]. Total carbohydrate content was determined in the precipitated EPS according to Dubois et al. [14] and the experiment was conducted in five replicates.

In Vitro Biocontrol Activity

Inhibitory activity of isolates PF23 and $PF23^{EPS-}$ against *M. phaseolina* was checked on DMM agar amended with different concentrations of NaCl (0–2,000 mM). Briefly, 6-mm mycelium disk of fungal pathogen was centrally placed on DMM plates, and 0.5 µl of exponentially grown cultures of PF23 and $PF23^{EPS-}$ were spot inoculated 1 cm away from the edge of the plate. The plates were incubated at 28 °C for 6 days and percentage inhibition was determined [4]. The 48 h culture of strain PF23 and $PF23^{EPS-}$ in DMM broth amended with different concentrations of NaCl (0–2,000 mM) was centrifuged at 7,826×*g* for 15 min followed by membrane filtration (0.45 µm, Merck, India), and antifungal activity of cell-free supernatant was detected by using the well diffusion method [24]. Briefly, spore suspension of *M. phaseolina* in 0.85 % saline was spread on DMM plates. A 6 mm well cut in DMM plates was filled with 50 µl of supernatant then incubated at 28 °C for 48 h to observe zone of inhibition in millimeter (mm)

In Vitro Antagonistic Activity of EPS

The antifungal activity of purified EPS obtained from PF23 (at varied salt concentrations ranging from 0 to 2,000 mM NaCl) was tested against *M. phaseolina* in separate flasks. For this, 500 µl spore suspension (OD 610 = 1.0) of *M. phaseolina* in 0.85 % saline and 100 µg of EPS (obtained at different NaCl concentrations) was added in 50 ml of DMM broth, and incubated at 28 °C for 7 days. In case of control, 500 µl of spore suspension was added in 50 ml of DMM broth. After 5 days, fungal mycelia were filtered (Whatman No. 1), oven dried at 70 °C, and percentage inhibition was calculated.

$$\text{Inhibition } \% = 1 - [\text{Dry weight of mycelium (sample)} / \text{Dry weight of mycelium (control)}] \times 100.$$

Composition of EPS

The precipitated EPS obtained from PF23 at different levels of salinity was hydrolyzed with 2 volumes of 2.5 M H_2SO_4 at 100 °C for 1 h. The solution was neutralized with 1 M sodium carbonate and spotted on the silica gel plate (Silica gel 60F 254; Merck). The plate was developed in a thin layer chromatography chamber using *n*-butanol:acetic acid:water (4:1:5 v/v) as the mobile phase at room temperature. The plate was dried, sprayed with alkaline potassium permanganate, and incubated at 100 °C for 10 min. The Rf values of the colored spots were measured and compared with those of standard carbohydrates (glucose, mannose, fructose, mannitol, arabinose, xylose, rhamnose, raffinose, galactose, and trehalose) [25].

In Planta Activity and Suppression of Charcoal Rot of Sunflower

The study was conducted to detect PGP response and charcoal rot suppression ability of EPS producing strain PF23 and negative strain $PF23^{EPS-}$ taking sunflower as the test crop under saline and non-saline conditions. Seeds of sunflower were surface sterilized for 2 min with 70 % ethanol followed by 2 % sodium hypochlorite (10 min). After thorough rinsing with sterile distilled water, the seeds were germinated in glass tubes of 50 ml capacity (2 seeds per tube), 1/3 filled with autoclaved plant growth media (PGM) (containing 1.2 mM K_2HPO_4, 0.4 mM KH_2PO_4, 5 mM $CaCl_2$, 3.35 mg ferric citrate $l^{-1,}$ 2.5 Mm $MgSO_4$, 2.5 mM K_2SO_4, 10 µM $MnSO_4$, 20 µM H_3BO_3, 5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 1.5 µM $CaSO_4$, 1.0 µM $NaMoO_4$, and 1 % agar, with pH 6.8) [18] supplemented with 125 mM NaCl (as sunflower seedlings displayed germination only up to 125 mM salinity level) and without NaCl in following sets of treatments: (i) non-bacterized seeds (ii) seeds treated with PF23 (iii) seeds + *M. phaseolina* (iv) seeds treated with PF23 + *M. phaseolina* (v) seeds + PF23^{EPS-} and (vi) seeds + PF23^{EPS-} + *M. phaseolina*. Seeds were bacterized by dipping in cell suspension of PF23 and PF23^{EPS-} (OD 610 = 0.1) for 10 min and dried overnight under aseptic conditions. *M. phaseolina* treatment was given by inoculating 10 µl of spore suspension in 0.85 % saline on the surface of PGM after 2 days of seed application [30]. The experiment was conducted in five replicates and germination percentage was calculated after 5 days according to Ranal and Santana [43]. The extent of infection by *M. phaseolina* was indicated by the presence of dark brown lesions on root systems. Percentage disease incidence was calculated as percentage of diseased plants out of the total number of plants.

In Vivo PGP Activity and Suppression of Charcoal Rot of Sunflower

Experiment was conducted in small plastic pots (24 × 12 × 12 cm) under controlled conditions (temperature 22 ± 2 °C, humidity 70–80 %, 12 h photoperiod) in plant growth chamber. Fungal inoculum was prepared on oat (*Avena sativa*) grains and added to the sterilized (autoclaved) sandy loam soil (pH 7.24) so as to get 10^4 fungal propagules/gm of soil before seed sowing (05 seeds/pot). Sunflower seeds were dipped for 10 min in a cell suspension of isolate PF23 and PF23^{EPS-} (OD 610 = 0.1) mixed with carboxy methyl cellulose (CMC, 1 %) and dried overnight in aseptic conditions [57]. The experiment was conducted in sterilized soil in similar sets of treatments as *in planta* study, taking five replicates. The effect of treatments was determined under control (received only normal irrigation water) and saline (irrigated as per requirement with 125 mM NaCl solution) conditions [41]. Five plants from each set were taken randomly to determine root associated soil/root tissue ratio. Plant watering was stopped 6 days before harvesting to facilitate the separation of root-associated soil from bulk soil. Roots with adhering soil were carefully separated from bulk soil by gentle agitation for 1 min. Root-associated soil was removed from root tissue by washing them in sterile water. Root-associated soil dry mass (dm) and root dm were measured after 24 h at 105 °C, to calculate root-associated soil/root tissue. Other plant growth parameters (shoot and root length, fresh weight, dry weight, head diameter, and seed yield) and disease incidence were measured after 120 days. Shoot length, root length, and head diameter were measured in centimeter (cm) soon after the plants were uprooted. Fresh weight was taken by uprooting the plant and rinsing with tap water until all visible sand and soil particles were removed. Particles that adhered strongly to the roots were manually removed with tweezers. After blotting the excess moisture from the plant with absorbent paper, the fresh weight was measured [10]. For dry weight, plants were placed into tarred aluminum foil pouches; samples were dried in an oven at 75 ± 2 °C for 16 h and weighed subsequently [10]. Whereas seed yield was calculated after separating the seeds from the head and weighing number of seeds per plant. Percentage disease incidence was calculated as percentage of diseased plants out of the total number of plants.

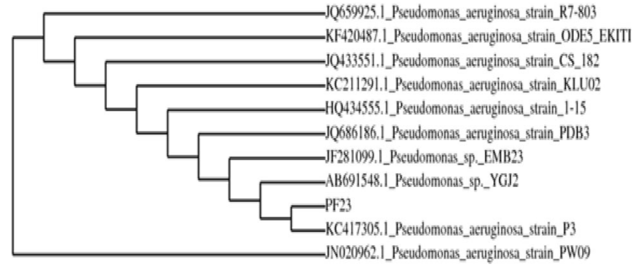

Fig. 1 Comparative sequence analysis of 16S rRNA gene from *P. aeruginosa* PF23 and representative related strains from GenBank

Statistical Analysis

The data generated during quantitative evaluation of EPS and seed germination or plant growth promotion values were analyzed by means of ANOVA, and means were compared by the Duncans Multiplicity Test Range (DMRT) using the SPSS software (ver. 10.1, SPSS Inc., www.spss.com). The significance level for all analysis was $P = 0.05$.

Results

Microorganisms and Growth Conditions

Isolate PF23 was selected from the collection of pseudomonads, as it displayed maximum stress tolerance capacity and high EPS production. Isolate PF23 was fluorescent, Gram-negative, motile rod, oxidase- and catalase-positive indicating according to Bergey's Manual of Systematic Bacteriology [22] to be a member of the *Pseudomonas* species. The comparison of complete 16S rRNA gene sequence (Fig. 1) of the isolate PF23 with the sequence of the other strains of the genus *Pseudomonas* showed 99 % relatedness to *Pseudomonas aeruginosa* (Gen Bank accession number AB691548.1 and KC417305.1). Based on biochemical, physiological characteristics, and nucleotide homology, isolate PF23 was identified as *Pseudomonas aeruginosa* PF23 and sequence data submitted to NCBI Gen Bank with accession number KF598858.

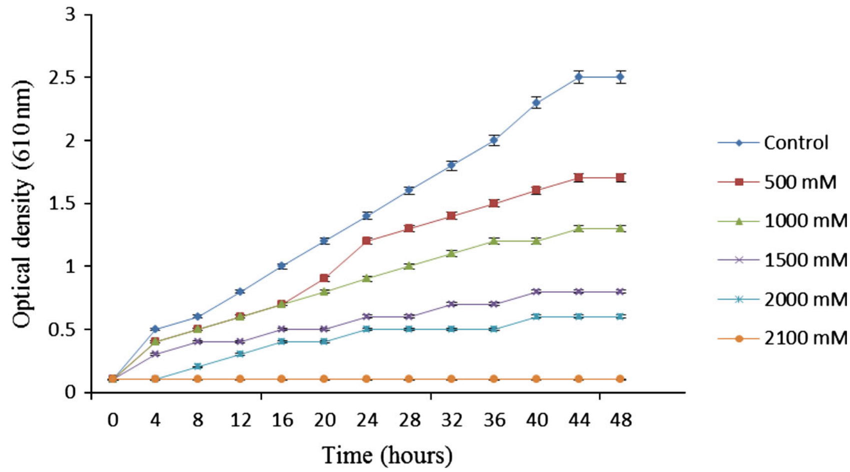

Fig. 2 Salinity tolerance assay of PF23. *Error bars* show the standard deviation of the mean values of five replicates. Five samples were analyzed for each replication, and each treatment consisted of five replications

Plant Growth Promoting Attributes

PF23 was positive for EPS, siderophore, and IAA production but negative for phosphate solubilization, HCN production, chitinase, β-1-3 glucanase, and nitrogen fixation. The production of IAA (in supernatant) and siderophore (on CAS agar plate) got drastically reduced with increase in salt concentration and was completely inhibited at 100 mM NaCl concentration. On the other hand, an increase in EPS production was recorded with progressive increase in salinity in precipitated supernatant.

Chemical Mutagenesis for Developing EPS-Defective Mutant

Of the 100 mutant clones, 6 were identified as defective for EPS production. Two of these mutants had stable mutations. EPS-defective stable mutant PF23^{EPS-} showed maximum reduction of 86 % in production of EPS in comparison with wild strain PF23.

Salinity Stress Assay

Isolate PF23 could tolerate salinity level up to 2,000 mM (12 %). Salt shock with 100 mM NaCl did not affect the growth, but higher osmotic stress of 500, 1,000, and 1,500 brought significant reduction in the optical density by 32, 48, and 68 %, respectively, in comparison with non-stress conditions (0 mM NaCl) (Fig. 2). No growth was observed above 2,000 mM salt concentration. On the other side EPS-defective strain PF23^{EPS-} displayed 80 % reduction in growth in presence of 200 mM NaCl, suggesting it to be a non-salt tolerating strain.

Effect of Saline Stress on EPS Production

Increase in osmotic stress or increase in salinity, brought increment in the EPS production up to a certain limit. In

Table 1 Effect of saline stress on EPS production

Salt stress (mM)	Dry weight of EPS (g/l)	EPS components	Carbohydrate in EPS (μg/mg^{-1})
0	0.801 ± 0.01^{a}	Glucose	126 ± 0.02^{a}
100	0.811 ± 0.02^{a}	Glucose, galactose	131 ± 0.01^{b}
500	0.901 ± 0.03^{ab}	Glucose, galactose	133 ± 0.03^{b}
1,000	1.010 ± 0.01^{b}	Glucose, rhamnose	138 ± 0.02^{c}
1,500	1.102 ± 0.02^{c}	Glucose, rhamnose, trehalose	145 ± 0.01^{d}
1,700	1.210 ± 0.01^{cd}	Glucose, mannose, rhamnose, trehalose	149 ± 0.03^{de}
2,000	1.323 ± 0.03^{d}	Glucose, mannose, rhamnose, trehalose	152 ± 0.02^{e}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference ($P = 0.05$) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replications

stress tolerating strain PF23, there was increase in EPS production by about 26, 38, and 66 % at salinity level of 1,000, 1,500, and 2,000, respectively (Table 1). EPS-defective mutant PF23^{EPS-} showed maximum EPS production at 0 mM NaCl (0.109 g/l) which drastically reduced above 100 mM NaCl, and finally got diminished beyond 200 mM NaCl, respectively.

In Vitro Biocontrol and Antagonistic Activity of EPS

Strain PF23 and its cell-free culture supernatant demonstrated 90 and 88 % inhibition of *M. phaseolina* at 0 mm NaCl concentration, respectively. Biocontrol ability of the strain was maintained up to 500 mM (54 and 51 % inhibition by cell and cell-free culture supernatant, respectively) salinity but thereafter inhibitory activity ceased.

PF23^{EPS-} and its cell-free culture supernatant displayed antifungal activity only under unstressed (0 mM) conditions and that was about 55 and 50 %, respectively. EPS obtained from PF23 at 100, 200, 300, 400, and 500 mM NaCl displayed strong antagonistic activity against *M. phaseolina* and resulted in 79, 72, 64, 55, and 41 % reduction in growth, respectively, in comparison with control. EPS obtained at 0 mM (control) antagonized *M. phaseolina* by 84 %.

Composition of EPS

Analysis of EPS constituents by thin layer chromatography revealed differences in the sugar components of salinity tolerant strain PF23 under non-stressed and stressed conditions. Under normal conditions (0 mM NaCl) glucose (Rf 0.42) was present as the saccharide unit in the EPS hydrosylate, whereas EPS obtained under salt stress was composed of glucose (Rf 0.42), galactose (Rf 0.37), rhamnose (Rf 0.74), mannose (Rf 0.46), and trehalose (Rf 0.32) (Table 1). Carbohydrate content of EPS progressively increased from 126 to152 μg/mg^{-1} with increase in salinity from 0 to 2,000 mM. EPS-defective strain PF23^{EPS-} contained only glucose as its saccharide unit both under control and maximum stress conditions (0–200 mM) and there was steep reduction in carbohydrate content from 20 μg/mg^{-1} to 5 μg/mg^{-1} under same conditions.

In Planta Activity and Suppression of Charcoal Rot of Sunflower

Seed biopriming with PF23 showed significant increase in germination % in comparison with non-primed seeds (control) both under saline (125 mM NaCl) and non-saline (0 mM) conditions. Seed biopriming brought 25 and 50 % increment in germination % in comparison with unprimed seeds under non-saline and saline conditions, respectively. Treatment with PF23 brought increment in root length, shoot length, fresh weight, dry weight by 105, 59, 115, 212 %, respectively, in comparison with control (untreated seeds) under saline stress. Under non-saline conditions it was observed that PF23 brought 100, 31, 46, and 117 % enhancement in root length, shoot length, fresh weight, and dry weight, respectively.

Strain PF23 displayed increase in plant growth parameters of sunflower in both the absence and the presence of pathogen, under saline and non-saline conditions. In presence of *M. phaseolina,* PF23 brought increment in fresh weight and dry weight by 103 and 84 % in comparison with unbacterized seeds, under saline conditions. In comparison with non-bacterized control, PF23 showed 80 and 70 % reduction of disease incidence in non-saline and

Table 2 Influence of *P. aeruginosa* PF23 treatment on growth of sunflower plants in both the absence and the presence of *M. phaseolina*, under non-saline (control) and saline (*in planta*) conditions

	Non-stress condition (0 mM NaCl)					Stress condition (125 mM NaCl)				
Treatment	Germination %	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Germination %	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)
Unbacterized seeds	80 ± 0.01^{c}	3.0 ± 0.02^{b}	11.5 ± 0.01^{b}	1.44 ± 0.02^{b}	0.24 ± 0.03^{b}	60 ± 0.02^{b}	2.0 ± 0.01^{b}	7.6 ± 0.02^{b}	0.75 ± 0.02^{b}	0.126 ± 0.01^{b}
PF23	100 ± 0.02^{e}	6.0 ± 0.01^{d}	15.1 ± 0.03^{d}	2.10 ± 0.01^{e}	0.52 ± 0.01^{c}	90 ± 0.01^{d}	4.1 ± 0.03^{d}	12.1 ± 0.01	1.61 ± 0.01^{d}	0.393 ± 0.03^{d}
PF23^{EPS-}	80 ± 0.03^{c}	3.4 ± 0.03^{b}	12.2 ± 0.01^{bc}	1.52 ± 0.03^{c}	0.26 ± 0.03^{b}	60 ± 0.03^{b}	2.1 ± 0.01^{b}	7.2 ± 0.03^{b}	0.71 ± 0.03^{b}	0.124 ± 0.02^{b}
M. phaseolina	60 ± 0.01^{a}	2.6 ± 0.01^{a}	08.0 ± 0.02^{a}	1.11 ± 0.01^{a}	0.098 ± 0.02^{a}	40 ± 0.01^{a}	1.0 ± 0.02^{a}	3.0 ± 0.01^{a}	0.60 ± 0.01^{a}	0.118 ± 0.01^{a}
PF23+ *M. phaseolina*	90 ± 0.02^{d}	5.0 ± 0.02^{c}	13.0 ± 0.01^{c}	1.61 ± 0.03^{d}	0.29 ± 0.01^{b}	80 ± 0.03^{c}	3.0 ± 0.03^{c}	9.0 ± 0.03^{c}	1.22 ± 0.02^{c}	0.217 ± 0.03^{c}
PF23^{EPS-} + *M. phaseolina*	70 ± 0.01^{b}	3.1 ± 0.01^{b}	11.0 ± 0.02^{b}	1.40 ± 0.03^{b}	0.21 ± 0.01^{b}	40 ± 0.01^{a}	1.2 ± 0.01^{a}	3.2 ± 0.01^{a}	0.62 ± 0.03^{a}	0.120 ± 0.02^{a}

Results are the mean ± SD ($n = 5$). Means in the columns followed by same superscript letters indicate no significant difference ($P = 0.05$) by Duncan's multiple range test. Two samples were analyzed for each replication, and each treatment consisted of five replications saline conditions, whereas EPS-defective strain $PF23^{EPS-}$ was significantly ineffective in suppressing disease under saline conditions. $PF23^{EPS-}$ displayed significantly similar results as obtained by control (untreated seeds), both under saline and non-saline conditions (Table 2).

In Vivo PGP Activity and Suppression of Charcoal Rot of Sunflower

Results of in vivo study showed that treatment of seeds with PF23 caused significant increase in root length, shoot length, dry weight, and head diameter under saline and non-saline conditions over untreated seeds and infested seeds in the presence as well as in the absence of phytopathogen (Table 3). Biopriming of seeds with PF23 brought 53 and 50 % increase in seed yield under non-stress and stress conditions, respectively, in comparison with unbacterized seeds.

M. phaseolina caused 79 and 81 % incidence of disease in non-saline and saline conditions. Strain PF23 resulted in 71 and 63 % reduction of disease incidence in comparison with non-bacterized control under non-saline and saline conditions, respectively. EPS-defective strain $PF23^{EPS-}$ brought reduction of disease incidence by 33 and 17 % under non-saline and saline conditions. In the presence of pathogen (*M. phaseolina*), strain PF23 caused 93 and 138 % enhancement in seed yield under non-saline and saline conditions, respectively, in comparison with *M. phaseolina*-infested untreated seeds. Treatment of seeds with $PF23^{EPS-}$ + *M. phaseolina* brought significant enhancement in dry weight, head diameter, and seed yield of sunflower by 72, 28, and 59 % in comparison with *M. phaseolina*-infested seeds only under non-saline state. However, the mutant was ineffective in controlling the pathogen under saline conditions and there was no EPS recorded as well (Table 3).

Discussion

Pseudomonas aeruginosa PF23 displayed high salt tolerance and EPS production with progressive increase in salinity from 100 to 2,000 mM NaCl. EPS-defective mutant $PF23^{EPS-}$ didn't displayed salt tolerance above 200 mM and showed 87 % reduction in EPS production in comparison with wild strain. Thus, a strong correlation could be observed between EPS production and salinity tolerance. At low salt concentration (up to 500 mM NaCl), PF23 displayed 0.901 g/l EPS that constituted mainly of glucose and galactose as its components. Whereas further increase in salinity (up to 2,000 mM NaCl) resulted in glucose, rhamnose, mannose, and trehalose as major constituents of EPS. Strain PF23, its cell-free culture

Table 3 Influence of *P. aeruginosa* PF23 treatment on growth of sunflower plants in both the absence and the presence of *M. phaseolina*, under non-saline (control) and saline (in vivo) conditions

	Non-stress conditions						Stress conditions					
Treatments	Root length (cm)	Shoot length (cm)	Dry weight (gm)	Head diameter (cm)	Seed yield (g/pot)	Root associated soil/root tissue	Root length (cm)	Shoot length (cm)	Dry weight (gm)	Head diameter (cm)	Seed yield (g/pot)	Root associated soil/root tissue
Unbacterized seeds	08.00 ± 0.03^b	50.3 ± 0.01^b	1.51 ± 0.02^b	6.32 ± 0.02^b	10.50 ± 0.02^b	0.553 ± 0.03^b	6.31 ± 0.01^b	45.1 ± 0.03^b	1.31 ± 0.01^b	5.01 ± 0.01^b	9.00 ± 0.01^b	0.321 ± 0.03^b
PF23	10.95 ± 0.01^d	62.1 ± 0.03^d	2.39 ± 0.01^d	8.10 ± 0.03^d	16.10 ± 0.03^e	1.231 ± 0.01^d	9.24 ± 0.03^d	59.8 ± 0.01^e	2.18 ± 0.03^d	7.45 ± 0.02^d	13.50 ± 0.03^d	1.021 ± 0.01^d
$PF23^{EPS-}$	08.50 ± 0.03^b	55.6 ± 0.01^b	1.58 ± 0.03^b	6.71 ± 0.01^b	11.70 ± 0.01^c	0.601 ± 0.03^c	6.32 ± 0.01^b	46.9 ± 0.03^c	1.34 ± 0.02^b	5.12 ± 0.03^b	9.30 ± 0.02^b	0.359 ± 0.03^b
M. phaseolina	06.17 ± 0.01^a	35.9 ± 0.02^a	0.89 ± 0.02^a	5.10 ± 0.03^a	07.11 ± 0.03^a	0.319 ± 0.02^a	5.22 ± 0.03^a	32.6 ± 0.02^a	0.68 ± 0.02^a	4.20 ± 0.01^a	5.00 ± 0.01^a	0.289 ± 0.01^a
PF23 + *M.phaseolina*	09.94 ± 0.02^c	59.9 ± 0.03^c	2.01 ± 0.01^c	7.50 ± 0.02^c	13.70 ± 0.02^d	1.189 ± 0.01^d	8.54 ± 0.02^c	56.2 ± 0.01^d	1.82 ± 0.03^c	7.01 ± 0.02^c	11.90 ± 0.03^c	0.949 ± 0.02^c
$PF23^{EPS-}$ + *M.phaseolina*	08.30 ± 0.03^b	53.1 ± 0.02^b	1.53 ± 0.03^b	6.50 ± 0.01^b	11.30 ± 0.01^c	0.591 ± 0.03^c	5.32 ± 0.01^a	33.7 ± 0.03^a	0.72 ± 0.01^a	4.50 ± 0.03^a	5.30 ± 0.02^a	0.339 ± 0.03^b

Results are the mean ± SD ($n = 5$). Means in the columns followed by same superscript letters indicate no significant difference ($P = 0.05$) by Duncan's multiple range test. Five samples were analyzed for each replication, and each treatment consisted of five replications supernatant and purified EPS displayed strong biocontrol potential against *M. phaseolina* up to 500 mM NaCl, suggesting the presence of bioactive compounds in EPS (mainly glucose and galactose) that function well at low level of salinity. Several workers reported the role of sugar analogs (2-deoxy-D-glucose) in inhibiting the growth of several yeasts and filamentous fungi [8, 9, 11, 16, 33]. Apart from it, El Ghaouth et al. [16, 17] reported the role of glucose in inhibiting the radial growth of several pathogens and causing morphological alterations in *Rhizopus stolonifer* and *Botrytis cinerea.* Although, the inhibitory activity of EPS in antagonizing, dreadful phytopathogens have been illustrated by several workers [3, 15, 38, 52] but its role under saline conditions is unknown so far.

Though, EPS actively participated in biological control of pathogens (up to 500 mM salinity), its role could also be related to stress amelioration. Addition of NaCl (above 500 mM) in the medium stimulated the mucoid growth (profuse spreading of the EPS) of the PF23 up to 2,000 mM. Increase in EPS production with increase in salinity suggests that under stress condition energy flow of the PF23 is directed toward protective mechanism, and synthesis of EPS is opted as a defensive strategy for maintaining its survivability and ameliorating salt stress [7, 44]. Discharge of EPS results in aggregation/flocculation and sheath formation around the cell, which significantly improves survival of bacterial cells in saline stress [27]. Variations in the saccharide composition with increase in salinity indicate structural differences among different polymers. EPS isolated during stressed conditions was composed of multiple saccharide units like rhamnose and trehalose. Role of mannose, rhamnose, and trehalose as stress ameliorators has been explained earlier also [13, 48]. Trehalose protects cellular enzymes by replacing water around macromolecules [58] and also stabilizes cell membranes during stress conditions [12, 13]. Under unfavorable conditions, synthesis of EPS with multiple sugars stabilizes cellular structure and offers protection from stress [21]. Thus, it may be suggested that by storing large quantities of carbon in the form of EPS during osmotic stress, PF23 cells may balance the osmotic pressure imposed by the environment and thus it (EPS) acts as a stress ameliorator or osmoprotective agent.

In planta and in vivo studies showed that seed biopriming with PF23 displayed significant increase in germination by 25 and 50 % in comparison with unprimed seeds under non-saline and saline conditions, respectively. Bioprimed seeds emerged at a more rapid rate than control. Seed biopriming with PF23 brought increment in plant growth parameters and yield under in vivo conditions, whereas EPS-defective strain did not significantly enhanced germination, plant growth parameters, and yield under saline conditions. Treatment of seeds with PF23^{EPS-} + *M. phaseolina* brought significant enhancement in plant growth parameter in comparison with *M. phaseolina* infested seeds only under non-saline state. However, the same treatment was ineffective in controlling the pathogen under saline conditions when there was no EPS recorded. Under non-stress conditions, PF23^{EPS-} brought increase in root length, shoot length, and seed yield in comparison with *M. phaseolina*-infested seeds, suggesting the role of other PGP metabolites like siderophore and IAA in growth promotion and yield enhancement, as the mutant was positive for these PGP characteristics. Whereas same mutant PF23^{EPS-} under stressed conditions was ineffective and unimpressive in enhancing plant growth parameters suggesting the absence of PGP activity under saline stress conditions, confirming the involvement of EPS in plant growth promotion under saline stress. Apart from plant growth promotion the role of EPS in suppression of charcoal rot disease could also be established by PF23 under saline and non-saline conditions. PF23 brought 70 and 63 % reduction of disease incidence in saline conditions both *in planta* and in vivo conditions. Bioprimed seeds (in *M. phaseolina* infested and non-infested treatment) brought significant increase in plant growth parameters like root length, shoot length, fresh weight, dry weight, and seed yield in comparison with control. The most conceivable reason for such heightened yield may be due to the fact that introduction of EPS strain brought significant increase in mass of root-associated soil/root tissue in comparison with uninoculated control as was clearly observed. Increased root associated soil/root tissue upsurges adhesion of soil particles, intensify soil aggregation, enhances soil texture, increase water holding capacity of soil, reduce water loss during stress conditions, and protect them from pathogenic invaders [45]. Increased root-associated soil on PF23 application showed that hydrophilization of soil leads to improved supply of nutrients that is responsible for plant growth promotion and disease suppression [1].

Microbial EPS released by PF23 also assisted in minimizing the effect of salt stress by functioning as an ameliorating agent. PF23 when introduced in soil releases EPS that acts as slimy, mucilaginous glue or cementing adhesive and assist in soil aggregation [1]. Aggregation of soil, influences organic matter storage, soil aeration, water infiltration, and mineral supply to plant thus playing significant role in fertility recapitalization and contributes to plant growth promotion [34]. Hence, multiple roles of EPS illustrated by the study proved that at lower concentrations EPS can function as a biocontrol metabolite that possesses antagonistic activity against dreadful phytopathogen *M. phaseolina.* As the concentration of EPS increased with progressive increase in salinity, it started to behave more as an osmoprotective agent and thus protected PF23 and sunflower from the plethora of osmotic stresses. Pandey et al. [39] also demonstrated that the endophytic *P. aeruginosa* isolated from wheat roots could confer biotic and abiotic stress tolerance in cucumber by modulating stress responses. Finally, when this EPS producing PF23 was introduced in salinized soil it served as a seed biopriming agent. It brought early emergence and germination of seedling, enhanced growth, and productivity of sunflower crop by ameliorating the effect of saline stress and suppressed the incidence of disease. Sarma and Saikia [50] reported the role of *P. aeruginosa* in alleviation of abiotic stresses in mung bean. Present study suggests applicability of *P. aeruginosa* PF23 in enhancing growth and production of sunflower crop in stress-affected regions.

It has been already assessed that more than 800 million hectare land throughout the world is suffering from salinization, and is responsible for 60 % loss in sunflower yield [42]. *P. aeruginosa* PF23 along with an important oil seed crop can thus also be used in reclamation of barren saline soils. Increase in production and reclamation of semiarid regions utilizing EPS-producing microbes can make great contribution in enhancing yields of stressed soils around the globe.

Conclusion

Isolate PF23 identified as *P. aeruginosa* displayed high salt tolerance and EPS production. PF23 acted as an excellent PGPR as well as biocontrol agent due to its capacity to produce EPS at high salt concentrations. EPS not only acted as a biocontrol metabolite but also as an osmoprotectant both for the host bacteria producing it as well as for the plant (sunflower). Inoculation of PF23 can serve as useful tool for decreasing salinity stress and enhancing the yield of sunflower crop in salt-affected soils. PGPRs such as *P. aeruginosa* PF23 can go a long way in enhancing crop yields in salinity-affected soils thus can be involved in reclamation of such habitats. Enhanced crop productivity from barren saline soils can be a key to the food security. However, further studies are required for designing stress tolerating bioformulation from EPS producing strain PF23 for promoting plant growth, protecting plant health, strengthening plant—microbe associations in stress-affected regions, protecting plant (sunflower) from the attack of phytopathogens and reclaiming arid and semiarid areas for increasing crop productivity.

Acknowledgments Thanks are due to Department of Biotechnology, Ministry of Science & Technology, New Delhi and Council of Science and Technology, Lucknow, India for financial support. Authors are grateful to Vice Chancellor, BBA University, Lucknow, India for support.

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Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (5): 73-81
Published online December 24, 2014 (http://www.cellmolbiol.com)
Received on May 25, 2014, Accepted on September 13, 2014.
doi : 10.14715/cmb/2014.60.5.13

Talc based exopolysaccharides formulation enhancing growth and production of *Hellianthus annuus* under saline conditions

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Abstract

Stress tolerating strain of *Pseudomonas aeruginosa* PF07 possessing plant growth promoting activity was screened for the production of exopolysaccharides (EPS). EPS production was monitored in the cell free culture supernatant (CFCS) and extracted EPS was further purified by thin layer chromatography. EPS producing cells were taken to design talc based formulation and its efficacy was checked on oilseed crop sunflower (*Hellianthus annuus*), under *in vivo* saline conditions (soil irrigated with 125 mM of saline water). Application of bioformulation significantly enhanced the yield and growth attributes of the plant in comparison to control (untreated seeds) under stress and non-stress conditions. Germination rate, plant length, dry weight and seed weight increased remarkably. The above findings suggest the application and benefits of utilizing EPS formulation in boosting early seedling emergence, enhancing plant growth parameters, increasing seed weight and mitigating stress in saline affected regions. Such bioformulation may enhance RAS/RT (Root Adhering Soil to Root Tissue ratio), texture of the soil, increase porosity, improve uptake of nutrients, and hence may be considered as commercially important formulation for renovation of stressed sites and enhancing plant growth.

Key words: Bioformulation, Exopolysaccharides (EPS), *Hellianthus annuus, Pseudomonas aeruginosa, Salinity.*

Introduction

Sunflower (*Helianthus annuus* L.) belonging to the family Asteraceae is an important oil seed crop grown in India (1). Oilseed crops, including sunflower have served as backbone of Indian economy since time immemorial, but nowadays the production is declining due to several abiotic stressors. Salinity is one of the major abiotic stress factor limiting plant growth and productivity (2). The total salt-affected land worldwide is estimated to be 900 million hectares, 6% of the total global land mass (3). In India about ten million-hectares of land is suffering from the constraint of salinization and Uttar Pradesh has about 1.28 million hectare saline soils (4, 5). Nowadays sunflower production is severely affected by several biotic and abiotic stresses. In fact salinity is one of the major stressors resulting in more than 60% loss in the sunflower production around the globe (6).

The extensive use of synthetic organic chemicals in the past decades has led to a number of long-term environmental problems. Repeated use of external inputs destroys the soil biota and reduces the nutritive value of soil, resulting in salinization which causes various stresses in agricultural plants (7). The need of the day is sustainable agriculture without harming the delicate balance of soil ecology as well as unlocking the mystery of biota influencing plant growth by using plant growth promoting rhizobacteria (PGPR) (8). The development of biological products based on beneficial microorganisms can extend the range of options for maintaining the healthy yield of crops under stress conditions such as salinity. In recent years, a new approach has been developed to alleviate salt stress in plants and that is by treating crop seeds and seedlings with tolerant PGPR strains (9).

Soil-borne fluorescent pseudomonads have received particular attention as PGPRs throughout the globe because of their catabolic versatility, excellent root colonizing ability and their capacity to produce a wide range of metabolites that favor the plant withstand under varied biotic conditions (10). Exopolysaccharides (EPS) produced by bacterial cells are instrumental in imparting stress tolerance to bacterial cell, but relatively little attention has been paid on this subject, particularly on EPS-producing fluorescent Pseudomonads and bioformulations developed from them (9). As the saline areas under agriculture have been on the rise every year across the globe, this is a matter of serious concern, but uptill now no possible remedy has been developed as regard to it (11). Thus delivering the stress tolerating PGPR, to the sunflower plant and soil in an effective way can go a long way in enhancing crop yields and remediation of saline soils.

Materials and methods

Bacterial strains

Bacterial strains were isolated from the rhizospheric region of the sunflower crop (*Helianthus annuus)* growing in semiarid conditions of west Kanpur, (20°38′ E and 80°21′ N; temperature maximum 48°C and minimum 1°C) (Uttar Pradesh, Northern India). Isolates were tested for morphological, physiological and biochemical characters according to Bergey's Manual of Systematic Bacteriology (12). On the basis of salinity stress tolerance capacities and plant growth promoting (PGP) qualities, isolate PF07 was selected and further identified by analysis of 1.5 kb 16S rRNA sequences. 16S gene sequence was queried for similarities with

BLAST (13) and with the Ribosomal Database Project (RDP) (14). Bacterial strain PF07 was identified as *Pseudomonas aeruginosa*, purified and maintained on stress tolerant Davis minimal medium (DMM) agar slants at 4˚C for further use (15,16). DMM was selected as it is a minimal media that provides conditions almost similar as faced by bacteria under natural stress environment.

Plant growth promoting attributes under saline conditions

PF07 was checked for plant growth promotory (PGP) attributes including phosphate solubilization, indole acetic acid (IAA) production, HCN production, siderophore production, chitinase, β-1-3 glucanase activity and EPS production abilities under saline (0 to 1600 mM NaCl) and non-saline conditions (0 mM NaCl). Phosphate solubilizing activity of PF07 was tested by spot inoculation on Pikovskaya's medium (17) and IAA production was detected in culture filtrate using salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) (18). HCN production was checked by observing the change in color of the filter paper impregnated with 0.5% picric acid in 1% Na_2CO_3 (19), whereas,

siderophore production was determined on Chrome-Azurol S medium according to Schwyn and Neilands (20). Extracellular chitinase activity was determined by spot inoculation on solid chitin minimal medium (CMM) whereas β-1,3 glucanase enzyme was assayed according to Dunne *et al*. (21), and EPS production was monitored by chilled ethanol precipitation method (22, 23).

Chemical mutagenesis for developing EPS-defective mutant

A loopful of PF07 cells were inoculated into 10 ml of DMM broth (16) and incubated at 28°C upto log phase. Subsequently 100 µg ml^{-1} 5-bromouracil was added and further incubated for 02 h. Cells were centrifuged, washed thrice with sterile water, and resuspended in 10 ml of DMM broth and mutants were fixed by overnight incubation at 28°C. The mutant bank was stored in 25% glycerol at -40°C (10). The clones of mutant bank were screened for saline tolerance and EPS production according to Sandhya *et al*. (23). The wild isolate $PF07^{EPS+}$ served as a control in the mutant screening test. Maximum EPS defective mutant was marked as $PF07^{EPS-}$. Mutagenic procedure worked effectively as EPS defective mutant $PF07^{EPS-}$ was repeatedly checked on DMM media for stability.

Salinity Tolerance Assay

The log phase cultures (OD610 = 0.1) of $PF07^{EPS+}$ and $PF07^{EPS-}$ were inoculated in DMM broth, amended with different concentrations of NaCl (0 to 1600 mM) and incubated at 28°C. Optical density was measured at 610 nm by spectrophotometer (GENESYS™6, Model, 335908-02) up to stationary phase (120 rpm). The experiment was conducted in five replicates.

Effect of salinity stress on EPS production

To determine the effect of saline stress (0-1600 mM) conditions on EPS content, selected isolate $PF07^{EPS+}$ and its mutant $PF07^{EPS-}$ were grown in DMM broth, with gradients of NaCl concentrations (0-1600 mM) for seven days. Cells were harvested by centrifugation (10 min at 11,000 x g). The supernatant was filtered through 0.45 µm nitrocellulose membrane, two volumes of cold ethanol were added to culture supernatants and stored overnight at 4°C. Precipitate was collected by centrifugation (20 min at 2,500 x g), suspended in demineralized water, and mixed with 2 volumes of cold ethanol. Samples were centrifuged (2,500 x g) and the pellets were dried at 100°C and weighed. The amount of EPS was expressed as polymer dry mass and expressed in g/l (22, 23).

Components of EPS

The precipitated EPS obtained from $PF07^{EPS+}$ at different levels of salinity was hydrolyzed with 2 volumes of 2.5 M H_2SO_4 at 100°C for 1 h. The solution was neutralized with 1 M sodium carbonate and spotted on the silica gel plate (Silica gel 60F 254; Merck). The plate was developed in a thin layer chromatography (TLC) chamber using n-butanol: acetic acid: water (4:1:5v/v) as the mobile phase at room temperature. The plate was dried, sprayed with alkaline potassium permanganate, and incubated at 100°C for 10 min. The Rf values of the colored spots were measured and compared with those of standard carbohydrates (glucose, mannose, fructose, mannitol, arabinose, xylose, rhamnose, raffinose, galactose) (24).

Determination of the carbohydrate content in the EPS

The carbohydrate concentrations were determined according to Gaudy's method (25). The precipitated EPS obtained from $PF07^{EPS+}$ at different levels of salinity (0 to 1600 mM NaCl) was hydrolyzed with 2 volumes of 2.5 M H_2SO_4 at 100°C for 1 h. Briefly, cell free culture supernatant (CFCS) was dissolved in phosphate buffer (1ml) and added to 10 ml sterile test tubes. Freshly prepared Anthrone solution (1ml) was added in each test tube. The mixture was incubated in a water bath at 95˚C for 15 min. After incubation, the mixture was allowed to cool to room temperature. Cooled aliquots (200 µl) were transferred to micro plate wells and read at 620 nm using an Elisa plate reader (Thermo scientific Multiskan Ex Type 355). Glucose was used as a standard to construct a standard curve.

Determination of the protein content in the EPS

Protein content, were determined by the modified method of Lowry (26). EPS (10 µl) was dissolved in phosphate buffer and inoculated into wells of a micro titter plate. Control wells were inoculated with phosphate buffer. Comassie plus reagent (300µl) was added to each well. The plate was incubated at room temperature for 10 min. After incubation, absorbance was read at 595 nm using an Elisa plate reader, (Thermo scientific Multiskan Ex Type 355). Bovine serum albumin (BSA) was used as a standard to construct the standard curve.

Talc-Based Formulation from EPS producing bacterial Cells

The talc-based formulation from $PF07^{EPS+}$ and $PF07^{EPS-}$ were developed by following the method described by Vidhyasekaran and Muthailan (27). In brief, one kilogram of talc powder was taken and pH adjusted to neutral by adding $CaCO_3$ at the rate of 15 g/kg. The 400 ml of 7 days grown bacterial suspension was mixed separately with carrier-cellulose mixture under aseptic conditions. After drying (approximately 35% moisture content) overnight under sterile conditions, it was packed in polypropylene bag, sealed, and stored at room temperature to determine population density for 180 days. The population density was measured by mixing 1 g of the bioformulation in 10 ml of distilled water aseptically and serially diluted to 10^{-6} and 10^{-7}. The prepared bioformulation from $PF07^{EPS+}$ and $PF07^{EPS-}$ were taken for coating sunflower seeds.

In vitro PGP activity

The study was conducted to detect PGP response ability of $PF07^{EPS+}$ and $PF07^{EPS-}$, taking sunflower as the test crop under saline and non-saline conditions. Seeds of sunflower were surface sterilized for 2 min with 70% ethanol followed by 2% sodium hypochlorite (10 min). Surface sterilized seeds of sunflower were soaked in suspension of talc-based formulations in sterile distilled water (SDW) (1:1 w/v) for 01 h, and then dried under shade (28). The seeds were germinated in glass tubes of 50 ml capacity (2 seeds per tube), 1/3 filled with autoclaved plant growth media (PGM) (containing 1.2 mM K_2HPO_4, 0.4 mM KH_2PO_4, 5 mM $CaCl_2$, 3.35 mg ferric citrate l^{-1}, 2.5 Mm Mg SO_4, 2.5 mM K_2SO_4, 10 µM $MnSO_4$, 20 µM H_3BO_3, 5µM $ZnSO_4$, 0.2 µM $CuSO_4$, 1.5 µM $CaSO_4$, 1.0 µM $NaMoO_4$, 1% agar, with pH 6.8) (29) supplemented with 125 mM NaCl (as sunflower seedlings displayed germination only up to 125 mM salinity level) and without NaCl in following sets of treatments: (i) non bacterized seeds (untreated seeds, control); (ii) seeds coated with talc formulation (unbacterized formulation) (iii) seeds treated with talc based formulation of $PF07^{EPS+}$; (iv) seeds treated with talc based formulation of $PF07^{EPS-}$.

In vivo (pot study) PGP Activity

Experiment was conducted in small plastic pots (24×12×12 cm) during the month of March-June in year 2012 and 2013 (for two consecutive years). The experiment was conducted in sterilized soil supplemented with 125 mM NaCl (as sunflower seedlings displayed germination only up to 125 mM salinity level) and without NaCl in similar sets of treatments as mentioned in *in planta* studies.

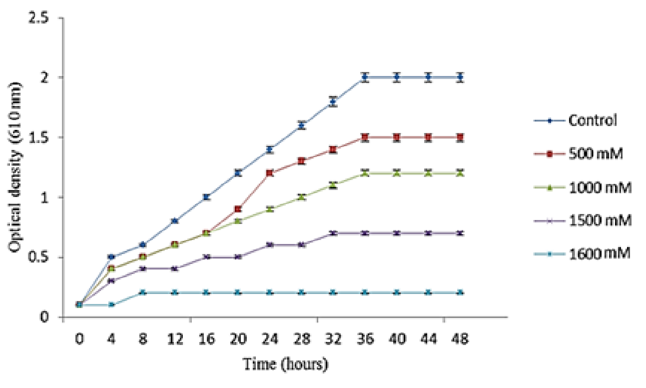

Figure 1. Salinity tolerance assay of PF07. Error bars show the standard deviation of the mean values of five replicates. Five samples were analyzed for each replication, and each treatment consisted of five replications.

The effect of treatments was determined under control (received only normal irrigation water) and saline (irrigated as per requirement with 125 mM NaCl solution) conditions (30). Five plants from each set were taken randomly to determine root associated soil / root tissue ratio (RAS/RT). Plant watering was stopped 6 days before harvesting to facilitate the separation of root associated soil from bulk soil. Roots with adhering soil were carefully separated from bulk soil by gentle agitation for 1 min. RAS was removed from RT by washing them in sterile water. RAS dry mass (dm) and RT dm were measured after 24 h at 105°C, to calculate RAS/RT. Other plant growth parameters including (shoot and root length, fresh weight, dry weight, head diameter and seed yield) and were measured after 120 days.

Statistical analysis

The data generated during quantitative evaluation of EPS and plant growth parameters were analyzed by means of analysis of variance (ANOVA), and means were compared by the Duncans Multiplicity Test Range (DMRT) using the SPSS software (ver. 10.1, SPSS Inc., www.spss.com). The significance level for the analysis was p=0.05.

Results

Bacterial strains

Isolate PF07 was selected from the collection of pseudomonads, as it displayed maximum salinity tolerance capacity and high EPS production. Isolate PF07 was fluorescent, Gram negative, motile rod, oxidase and catalase positive indicating according to Bergey's Manual of Systematic Bacteriology (12) to be a member of the genus *Pseudomonas*. Based on biochemical, physiological characteristics, and nucleotide homology, isolate PF07 displayed maximum relatedness to *Pseudomonas aeruginosa*.

Plant growth promoting attributes

PF07 was positive for siderophore, IAA production but negative for phosphate solubilization, HCN production, chitinase and β-1-3 glucanase. The production of IAA (in supernatant) and siderophore (on CAS agar plate) got drastically reduced with increase in salt concentration and was completely inhibited at 100 mM NaCl concentration. On the other hand an increase in EPS production was recorded with progressive increase in salinity in precipitated supernatant upto 1600 mM NaCl.

Chemical mutagenesis for developing EPS - defective mutant

Of the 100 mutant clones, 5 were identified as defective for EPS production. One of these mutants had stable mutation. EPS defective mutant $PF07^{EPS-}$ showed reduction of 92% in EPS production in comparison to wild strain $PF07^{EPS+}$. However, all the other PGP characters including IAA and siderophore were significantly similar to the wild strains.

Salinity tolerance Assay

Isolate PF07 $^{EPS+}$ could tolerate salinity level upto 1600 mM (9.6 %). Salt shock with 100 mM NaCl did not

Table 1. Effect of saline stress on EPS production, carbohydrate content and protein content.

Salt stress (mM)	Dry weight of EPS (g/l)	Sugar components present in EPS	Carbohydrate content (µg/ml^{-1})	Protein content (µg/ml^{-1})
0	0.821 ±0.01^{a}	Glucose	120±0.01^{a}	2230±0.01^{a}
100	0.834 ±0.02^{a}	Glucose, galactose	130±0.01^{ab}	2398±0.01^{b}
500	0.999 ±0.03^{ab}	Glucose, galactose	149±0.01^{b}	2471±0.01^{c}
1000	1.197 ±0.01^{b}	Glucose, rhamnose	152±0.01^{cd}	2507±0.01^{d}
1500	1.252 ±0.02^{c}	Glucose, rhamnose, trehalose	163±0.01^{de}	2596±0.01^{e}
1600	1.298 ±0.01^{cd}	Glucose, rhamnose, trehalose	175±0.01^{e}	2660±0.01^{f}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range test Two samples were analyzed for each replication, and each treatment consisted of five replications.

affect the growth, but higher osmotic stress of 500 mM, 1000 mM and 1500 mM brought significant reduction in the optical density by 33.33%, 66.66% and 185.71% respectively, in comparison to non-stress conditions (0 mM NaCl) (Fig. 1). No growth was observed above 1600 mM salt concentration. On the other side EPS defective strain PF07^{EPS-} displayed 82% growth reduction in presence of 150 mM NaCl, suggesting it to be a non-salt tolerating strain.

Effect of saline stress on EPS production

Increase in salinity brought increment in the EPS production up to a certain limit. Under saline conditions PF07^{EPS+} brought increase in EPS production by 21.68 %, 45.79 %, 52.49% and 58.09% at salinity level of 500 mM, 1000 mM, 1500 mM and 1600 mM, respectively (Table 1). PF07^{EPS+} produced 0.821 g/l of EPS at 0 mM salinity. PF07^{EPS-} displayed 0.016 g/l of EPS production at 0 mM salinity, which drastically reduced above 100 mM NaCl, and finally got diminished beyond 200 mM NaCl, respectively.

Components of EPS

Analysis of EPS constituents by TLC revealed differences in the sugar components of salinity tolerant strain PF07^{EPS+} under non-stressed and stressed conditions. Under normal conditions (0 mM NaCl) glucose (Rf 0.42) was present as the saccharide unit in the EPS hydrosylate, whereas, EPS obtained under salt stress was composed of various units including glucose (Rf 0.42), galactose (Rf 0.37), rhamnose (Rf 0.74) and trehalose (Rf 0.32) (Table 1).

Determination of the carbohydrate content in the EPS

There was an increase in the concentration of carbohydrates in EPS with the corresponding increase in NaCl level upto 1600 mM NaCl. Under saline conditions PF07^{EPS+} brought increment in carbohydrate content by 8.3%, 24.16%, 26.7%, 35.83%, 45.83% at 100 mM, 500 mM, 1000 mM, 1500 mM and 1600 mM NaCl, respectively in comparison to control (0 mM NaCl) (Table1).

Determination of the protein content in the EPS

Protein content increased remarkably with progressive increase in salinity. Protein content varied from 2130 µg/ml^{-1} at 0 mM NaCl to 3160 µg/ml^{-1} at 1600 mM NaCl. There was 7.88%, 16%, 18.29%, 35.96% and 48.35% increment in protein content (in PF07^{EPS+}) at 100 mM, 500 mM, 1000 mM, 1500 mM and 1600 mM NaCl, respectively, in comparison to control (0 mM NaCl) (Table 1).

Talc-Based Formulation from EPS producing Bacterial Cells

Talc based formulation showed 12.5%, 18.75%, 25%, 31.25% and 37.5% reduction in bacterial population after 30, 60, 90, 120 and 150 days of formualtion storage, respectively colony forming units (cfu) count after six month storage showed 42.5% reduction in PF07^{EPS+} population in talc based formualtion. Wheareasmutant strain PF07^{EPS-} brought 71.4% reduction in population density of bacterial cells, after six months storage (Fig. 2).

In planta PGP activity

Treatment of seeds with talc based bioformulation of EPS producing cells (PF07^{EPS+}) showed significant increase in germination % in comparison to EPS mutant cells (PF07^{EPS-}) especially under saline (125 mM NaCl) conditions. Talc based bioformulation of PF07^{EPS+} brought 30% and 50% increment in germination % in comparison to mutant PF07^{EPS-} cells under non - saline and saline conditions, respectively. Treatment by PF07^{EPS+} formulation brought increment in root length, shoot length, fresh weight and dry weight by 163.1%, 69.3%, 102.5% and 198.4% respectively, in comparison to PF07^{EPS-} cells under saline conditions. Under non-saline conditions it was observed that PF07^{EPS+} formulation brought 37.5% and 90.32% enhancement in root length and dry weight, respectively in comparison to PF07^{EPS-} bioformulation (Table 2). Whereas, under saline conditions, untreated (control) seeds and bioformu-

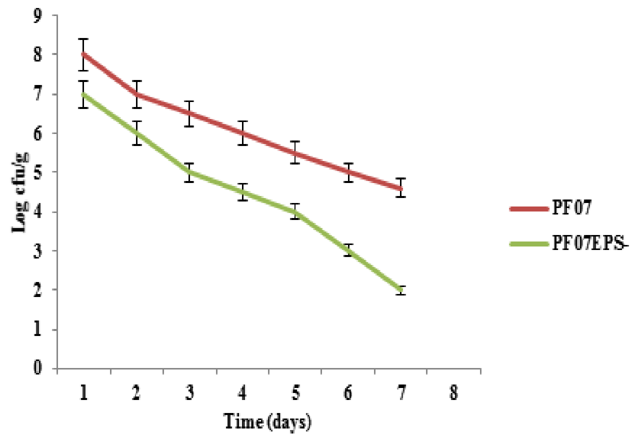

Figure 2. Population density of PF07 in talc based bioformulations on six month storage. Error bars show the standard deviation of the mean values of five replicates. Five samples were analyzed for each replication, and each treatment consisted of five replications.

Table 2. Influence of *P. aeruginosa* PF07 treatment on growth of sunflower plants in non-saline (control) and saline (*in planta*) conditions.

	Non-stress condition (0 mM NaCl)					Stress condition (125 mM NaCl)				
Treatment	**Germination %**	**Root length (cm)**	**Shoot length (cm)**	**Fresh weight (g)**	**Dry weight (g)**	**Germination %**	**Root length (cm)**	**Shoot length (cm)**	**Fresh weight (g)**	**Dry weight (g)**
Control (untreated seeds)	60±0.01^{a}	3.0 ±0.02^{a}	11.5 ±0.01^{a}	1.44±0.02^{a}	0.24 ±0.03^{a}	40 ±0.02^{a}	1.7±0.01^{a}	7.6±0.02^{a}	0.75±0.02^{a}	0.126 ±0.01^{a}
Talc coated seeds	60±0.01^{a}	2.9 ±0.02^{a}	11.4 ±0.01^{a}	1.42±0.02^{a}	0.23 ±0.03^{a}	40 ±0.02^{a}	1.6±0.01^{a}	7.5±0.02^{a}	0.74±0.02^{a}	0.125 ±0.01^{a}
PF07^{EPS-} formulation	70±0.02^{b}	4.0 ±0.01^{b}	12.1±0.03^{b}	1.56±0.02^{b}	0.31 ±0.01^{b}	40 ±0.01^{a}	1.9±0.03^{a}	7.8±0.01^{a}	0.77±0.01^{a}	0.128 ±0.03^{a}
PF07^{EPS+} formulation	100 ±0.02^{c}	5.5±0.01^{c}	14.3±0.03^{c}	1.79 ±0.01^{c}	0.59±0.01^{c}	90±0.01^{b}	5.0±0.03^{b}	13.2±0.03^{b}	1.56±0.01^{b}	0.382±0.01^{b}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range testTwo samples were analyzed for each replication, and each treatment consisted of five replications.

Table 3. Influence of *P. aeruginosa* PF07 treatment on growth of sunflower plants in under non-saline (control) and saline (*in vivo*) conditions.

	Non stress conditions (0 mM)						Saline stress conditions (125 mM)					
Treatments	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (gm)**	**Head diameter (cm)**	**Seed yield (g/pot)**	**RAS/RT**	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (gm)**	**Head diameter (cm)**	**Seed yield (g/pot)**	**RAS/RT**
Control (untreated seeds)	7.11±0.03^{a}	48.5±0.01^{a}	1.47±0.02^{a}	5.71±0.01^{a}	09.38±0.01^{a}	0.756±0.02^{a}	4.82±0.02^{a}	37.2±0.01^{a}	1.29±0.02^{a}	4.78±0.01^{a}	7.88±0.01^{a}	0.579±0.02^{a}
Talc coated seeds	7.10±0.03^{a}	48.6±0.01^{a}	1.48±0.02^{a}	5.72±0.01^{a}	09.39±0.01^{a}	0.753±0.02^{a}	4.81±0.02^{a}	37.0±0.01^{a}	1.30±0.02^{a}	4.77±0.01^{a}	7.86±0.01^{a}	0.577±0.02^{a}
PF07^{EPS-} formulation	7.39±0.01^{b}	51.6±0.02^{b}	1.50±0.01^{b}	6.18±0.03^{b}	10.03±0.02^{b}	0.771±0.01^{b}	4.85±0.03^{a}	38.5±0.02^{a}	1.30±0.01^{a}	4.80±0.03^{a}	7.90±0.02^{a}	0.581±0.03^{a}
PF07^{EPS+} formulation	8.61±0.02^{c}	56.9±0.03^{c}	1.59±0.03^{c}	6.87±0.02^{c}	11.56±0.03^{c}	0.999±0.03^{c}	6.72±0.01^{b}	43.4±0.03^{b}	1.40±0.03^{b}	5.59±0.02^{b}	10.91±0.01^{b}	0.859±0.01^{b}

Results are the mean ± SD (n = 5). Means in the columns followed by same superscript letters indicate no significant difference (p = 0.05) by Duncan's multiple range test. Five samples were analyed for each replication, and each treatment consisted of five replications.

lation of EPS mutant PF07^{EPS-} gave significantly similar results.

In vivo (pot study) PGP Activity

Results of *in vivo* study showed that treatment of seeds with PF07^{EPS+} bioformulation brought significant increase in root length, shoot length, dry weight, RAS/RT ratio and head diameter under saline and non - saline conditions over untreated seeds and EPS mutant cells PF07^{EPS-}. Biofomulation of PF07^{EPS+} brought increment in root length, seed yield and head diameter by 39.42%, 26.96% and 16.94% respectively in comparison to untreated seeds under saline conditions**.** RAS/RT ratio increased by about 47.84 % and 32.14 % under saline and non- saline conditions respectively, in comparison to unbacterized seeds (Table 3). Untreated seeds and bioformulation of EPS mutant PF07^{EPS-} gave significantly similar results under saline stress conditions.

Discussion

Isolate PF07^{EPS+} selected from the collection of fluorescent pseudomonads and identified as *P. aeruginosa*, displayed high salt tolerance and EPS production. EPS defective mutant PF07^{EPS-} didn't display salt tolerance above 150 mM and showed 92% reduction in EPS production in comparison to wild strain. There was remarkable increment of about 58% in EPS production with progressive increase in salinity from 100 mM to 1600 mM NaCl. Similarly there was significant increment in carbohydrate and protein content with increase in salinity. Protein content of PF07^{EPS+} increased by 48.35%, suggesting the induction or over expression of stress proteins in strains PF07^{EPS+} that assist in membrane stabilization. Several workers reported the accumulation of stress proteins under saline conditions, that protect the cells by balancing osmotic strength of bacterial membrane (31, 32, 33). It may also be speculated that as protein content increases it upsurges osmotic regulatory mechanisms, which in turn cause decreased sodium toxicity in cytoplasm, thereby protecting cells from salt shock. Sandhya *et al*. (34) reported accumulation of free amino acids and protein molecules in bacterial cells under osmotic stress. Prokaryotic cells respond to environmental stress by inducing specific sets of proteins characteristic to each stress. The proteins in each set of their coding genes constitute a stimulon, such as in oxidative and ionic stress (35).

Addition of NaCl (above 500 mM) in the medium also stimulated the mucoid, slimy growth (profuse spreading of the EPS) of the PF07^{EPS+} up to 1600 mM. Increase in EPS production with increase in salinity suggests that under stress condition energy flow of the PF07^{EPS+} is directed towards protective mechanism, and synthesis of EPS is opted as a defensive strategy for maintaining its survivability and ameliorating salt stress by bacterial cells (36). At low salt concentration (upto 500 mM NaCl) EPS mainly constituted glucose and galactose as its components. Whereas, further increase in salinity resulted in glucose, rhamnose and trehalose as major subunits. These subunits function as a carbon reservoir, which protect microorganisms from saline stress and fluctuations in water potential by enhancing water retention and regulating the diffusion of carbon sources in microbial environment (23). Thus, a strong relationship could be observed in between EPS production, protein content and salinity tolerance in PF07^{EPS+} cells.

For a bioformulation to have a high shelf life and be consistent, it must tolerate the constantly changing and frequently stressful environmental conditions. Developed talc-based bioformulation of PF07^{EPS+} was able to support population density of about 57.5% after six month storage whereas there was steep reduction in cell number of PF07^{EPS-} mutant by 71.4%. This also proved the impact and role of EPS in maintaining the cfu and protecting the cells in the formulation. Many microorganisms produce EPS as a strategy for growing, adhering to solid surfaces, and surviving adverse conditions.

Coating of seeds with cell free talc formulation reduced germination and plant growth parameters under saline conditions. Several workers reported decline in germination rate and plant growth parameters with increase in salinity due to reduction of the water passage into the seeds during imbibition (37, 38, 39, 40, 41) and due to slowing down the water absorption by the plant (42, 43, 44). Under non-stress conditions, formulation of PF07^{EPS-} brought significant increase in root length, shoot length and seed yield, suggesting the role of PGP metabolites like siderophore and IAA in growth promotion and yield enhancement, as the mutant was positive for these PGP characteristics. However, PF07^{EPS-} was ineffective and insignificant in enhancing plant growth parameters under saline conditions. Talc based formulation of PF07^{EPS+} was effective both under saline and non-saline conditions suggesting absence of PGP activity under saline stress conditions, and thus confirming the involvement of EPS in plant growth promotion under saline stress. In fact isolate PF07^{EPS+} was much better in enhancing the growth parameters of sunflower in *in vitro* and in *in vivo* studies under non-saline conditions, which was significantly higher than the mutant. Under saline conditions the enhancement in dry weight and yield was even starker as compared to control, the mutant being almost insignificant. Ashraf *et al.* (36) reported the significant enhancement in plant growth parameters in sunflower seedling by treating with EPS producing bacterial strains. The most conceivable reason for such heightened yield may be due to the fact that introduction of designed bioformulation brought significant increase in mass of RAS/RT in comparison to uninoculated control, as was clearly observed. Increased RAS/RT upsurges adhesion of soil particles, intensify soil aggregation, enhances soil texture, increase water holding capacity of soil and, reduce water loss during stress conditions (45). Increased root associated soil on PF07^{EPS+} application showed that hydrophilization of soil leads to improved supply of nutrients that is responsible for plant growth promotion (9).

Presence of carbohydrates and proteins in EPS provides a self-protective strategy to bacterial cells that help them to maintain their population density in soil after inoculation. Proteins released by PF07^{EPS+} generally deposit in plants grown under stress conditions and they may supply a storage form of nitrogen which play a vital role in osmotic adjustment, that is utilized during saline stress (46). Bioformulation designed from EPS producing *P. aeruginosa* PF07^{EPS+} displayed significant enhancement in plant growth producing attributes. EPS can bind soil particles to form microaggregates and macroaggregates. Plant roots fit in the pores between microaggregates and thus stabilize macroaggregates. Plants treated with EPS producing bacteria display increased resistance to saline stress due to improved soil structure (9, 23). EPS can also bind to cations including Na^+ thus making it unavailable to plants under saline conditions. Several workers reported the role of EPS produced by *Paenibacillus polymyxa* in increasing the aggregation of RAS/RT ratio on wheat (47, 9, 48). RAS forms the immediate environment where plants take up water and nutrients for their growth (9).

It has been already assessed that more than 800 million hectares land throughout the world is suffering from salinization, and is responsible for 60% loss in sunflower yield (49). *P. aeruginosa* PF07 $^{EPS+}$ along with an important oil seed crop can thus also be used in reclamation of barren saline soils. Increase in production and reclamation of semiarid regions utilizing EPS producing microbes can make great contribution in enhancing yield and production of sunflower in stressed soils around the globe.

Talc based formulation of the isolate PF07 $^{EPS+}$ brought more significant enhancement in plant growth parameters in comparison to non-formulated seeds and control seeds. The present investigation may be a step towards field application and commercialization of talc as a carrier for long-term sustenance and storage of stress tolerating PF07^{EPS+}, which may minimize salinity disposal in the environment. Conclusively, the talc provides prolong shelf life, and sustain the efficacy of the PF07^{EPS+} suggesting the carrier is stable for the bacteria. Talc based formulation was effective under saline conditions as it increased, yield of sunflower crop under saline conditions.

Thus, salt - tolerating PGPR PF07^{EPS+} and its bioformulation can serve as a nonpolluting and more cost-effective way to improve production in a saline stressed habitat. EPS helped not only in protecting the bacterial cells under saline conditions but also helped in maintaining high cfu in talc based formulation. The EPS based talc based formulation is thus recommended for enhancing the yield of sunflower in arid saline soils.

Acknowledgment
Authors are thankful to Prof R. C. Sobti, Vice Chancellor, BBA University, Lucknow, and Uttar Pradesh, India for his relentless support and encouragement.

Other articles in this theme issue include references (50-65).

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Journal of Pharmaceutical, Chemical and Biological Sciences
ISSN: 2348-7658
Jun-August 2014; 2(2):143-149
Available online at http://www.jpcbs.info

Original Research Article

Comparative study of different Carriers inoculated with Nodule forming and Free living plant growth promoting bacteria suitable for Sustainable Agriculture

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Received: 18 July 2014 Revised: 25 July 2014 Accepted: 26 July 2014

ABSTRACT

Rhizobium and *Pseudomonas* are bacteria that's are able to increase plant growth and provide nutrients to them in any condition even in stress condition and also have different plant growth characteristics such solubilized the minerals, fix the nitrogen and also chelate the inorganic compound for example iron and make it utilizable to plants thus making it beneficial as microbial biofertilizer and known it plant growth promoting rhizobacteria. The aim of this study was to determine potential five different carrier material for survival of PGPR (*Rhizobium* and *pseudomonas* strain) isolated from *Trigonella foenum Graecum* at room temperature for 8 weeks. Samples from the carrier materials (Sterilized and Non-sterilized) were taken every week and tested for the survivability and sustainability of the two different PGPR in it by determining viable cell count ($CFUg^{-1}$). The result showed that after eight weeks of storage treatment of carrier Coriander husk, saw dust and Begasse stored at room temperature (25-28^{o}C) was able to sustain the highest viable cell number of Co inoculation of *Rhizobia* and *Pseudomonas* followed by individual. These two carrier also had acceptable changes in pH value and moisture content followed by wood ashes and sand.

Keywords: Suitable Carriers; formulation; nodule forming bacteria; free living bacteria; sustainable agriculture

INTRODUCTION

Rhizobium and *Pseudomonas* both species are suitable known bacteria to be used as potential microbial inoculants or bio-fertilizer and bio-pesticides [1]. The microbial inoculants peculiarly those of rhizobacteria interact with both plant root and soil thus provide favourable effect on the plant growth and this was termed as plant growth promoting rhizobacteria (PGPR) [2-4]. The use of microbial inoculants as a bio-fertilizer increase crop yield, environment-friendly and can be utilized as an alternative or to reduce the usage of inorganic nitrogen fertilizer [5] inoculation of microbes,

Plant growth promoting fluorescent Pseudomonas enhancing growth of sunflower crop

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Abstract

Ten bacterial isolates were obtained from the rhizosphere of sunflower crop grown in the semi-arid region of west Kanpur. Isolates were further characterized on the basis of morphological, biochemical and physiological characteristics suggesting them to be the member of group fluorescent pseudomonas. Isolates were further monitored for plant growth promoting traits including IAA, phosphate solubilization, siderophore, nitrogen fixation, HCN, chitinase and β-1-3 glucanase activity. Amongst all the isolates, PF17 displayed maximum PGP attributes hence it was selected for doing further in vivo pot study taking sunflower as a test crop. Seed treatment with fluorescent pseudomonas PF17 brought enhancement in root length, shoot length, dry weight and seed yield of sunflower crop in comparison to control (untreated seeds). Hence it might be concluded from the study that fluorescent pseudomonas PF17 contains large number of PGP attributes, and its application contributed in enhancement of sunflower growth leading to better yield. In addition, ability to enhance growth of sunflower with the help of biological means appears to be of great ecological and economic importance.

Keywords: *PGPR, Pseudomonas, sunflower, phosphate solubilization, siderophore*

1. Introduction

Fertilizers and chemicals are vital components of modern agriculture because they provide essential plant nutrients. However, overuse of fertilizers can cause unanticipated environmental impacts. One potential way to decrease negative environmental impacts resulting from continued use of chemical fertilizers is inoculation of seeds with plant growth-promoting rhizobacteria (PGPR) (Adesemoye *et al.,* 2009)[1].

PGPRs exert beneficial effects on plant growth and development, and many different genera have been commercialized for use in agriculture. The genus *Pseudomonas*, comprises one of the most diverse and ecologically fit groups of bacteria on this planet, whose members are collectively referred to by the generic term Pseudomonad's. Members of the genus *Pseudomonas* are the ubiquitous and important component of the soil and rhizospheric ecosystem, where they play multifarious roles such as the recycling of organic matter, promotion of plant growth, alleviation of abiotic stress effects in plants and degradation of xenobiotic compounds (Arora *et al.,* 2013)[2]. The versatility and ecological fitness of this genus has been often attributed to its metabolic versatility and its ability to produce plant growth promoting (PGP) molecules, thereby gaining a niche advantage in the rhizosphere (Selvakumar *et al.,* 2015)[19].

Sunflower (*Helianthus annuus* L.) is a high yielding oilseed crop, but under scarce conditions, the yield is very lower than its real potential (Tewari and Arora 2014)[23]. Among the factors responsible for the low yield, imbalance use of fertilizers, improper plant protection, poor growth and sub optimum plant population are rather important (Moeinzadeh *et al.,* 2010)[16]. Suboptimum plant population generally results from poor and erratic germination. In recent years, a lot of studies have been done on enhancing germination and growth of seeds (Basra *et al.,* 2003)[3]. Seed treatment with beneficial PGPR is now a widely used commercial process that

World J Microbiol Biotechnol
DOI 10.1007/s11274-012-1112-4

ORIGINAL PAPER

Integrated approach for disease management and growth enhancement of *Sesamum indicum* L. utilizing *Azotobacter chroococcum* TRA2 and chemical fertilizer

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Received: 5 April 2012 / Accepted: 14 June 2012

Abstract *Azotobacter chroococcum* TRA2, an isolate of wheat rhizosphere displayed plant growth promoting attributes including indole acetic acid, HCN, siderophore production, solubilization of inorganic phosphate and fixation of atmospheric nitrogen. In addition, it showed strong antagonistic effect against *Macrophomina phaseolina* and *Fusarium oxysporum.* It also caused degradation and digestion of cell wall components, resulting in hyphal perforations, empty cell (halo) formation, shrinking and lysis of fungal mycelia along with significant degeneration of conidia. Fertilizer adaptive variant strain of *A. chroococcum* TRA2 was studied with *Tn5* induced streptomycin resistant transconjugants of wild type tetracycline-resistant TRA2 (designated TRA2$^{tetra+strep+}$) after different durations. The strain was significantly competent in rhizosphere, as its population increased by 15.29 % in rhizosphere of *Sesamum indicum*. Seed bacterization with the strain TRA2 resulted in significant increase in vegetative growth parameters and yield of sesame over the non-bacterized seeds. However, application of TRA2 with half dose of fertilizers showed sesame yield almost similar to that obtained by full dose treatment. Moreover, the oil yield increased by 24.20 %, while protein yield increased by 35.92 % in treatment receiving half dose of fertilizer along with TRA2 bacterized seeds, as compared to untreated control.

Keywords *Azotobacter chroococcum* · Antagonism · *Fusarium oxysporum* · *Macrophomina phaseolina* · PGPR · *Sesamum indicum*

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Introduction

Sesame (Sesamum indicum L.) is an important oilseed crop, usually rich in oil (38–54 %) and protein (18–20 %) originating in East Africa and India (Bedigian 1985). India ranks first in area (29 %), production (26 %) and export (40 %) of sesame in the world (Duhoon et al. 2004). The cultivated area of sesame increased markedly during the last few years while, the productivity has not increased by the same relative (Habbasha et al. 2007).

In Indian agricultural set up, recommended dose of chemical fertilizer for sesame crop is 120 kg ha^{-1} nitrogen, in three split doses of urea, 30 kg ha^{-1} phosphate in the form of diammonium phosphate (DAP) and 30 kg ha^{-1} potassium in the form of muriate of potash (MoP) in single dose.

In intensive cropping system, supplementing soil nutrients by use of chemical fertilizer is considered inevitable for obtaining optimum yield. Continuous use of chemical fertilizers subverts the soil ecology, disrupt environment, degrade soil fertility and consequently show harmful effects on human health (Ayala and Rao 2002) along with contaminating ground water (Joshi et al. 2006).

The need of the day is sustainable agriculture without harming the delicate balance of soil ecology as well as unlocking the mystery of biota influencing plant growth by using plant growth promoting rhizobacteria (PGPR). PGPR

Published online: 25 July 2012

Climate Change and Environmental Sustainability (October 2013) 1(2): 95-103
DOI: 10.5958/j.2320-642X.1.2.009

REVIEW ARTICLE

Plant growth promoting rhizobacteria for ameliorating abiotic stresses triggered due to climatic variability

Sakshi Tewari • Naveen Kumar Arora*

Abstract The world is gifted with varied landforms and diversity of climatic conditions such as the lofty mountains, the riverine deltas, high altitude forests, peninsular plateaus, variety of geological formations endowed with varying temperature and rainfall. Physical features like temperature, pH, salinity, water content constitute abiotic factors of the ecosystem and if these factors exceed beyond the threshold limit then, they might result in abiotic stresses. Economy of different countries relies on agriculture and abiotic stresses are the major constraints that limit crop productivity around the globe. The development of stress tolerant crops is not an easy and economical approach for sustainable agriculture; however microbial inoculation to alleviate stress tolerance is a better option because it minimizes production costs and environmental hazards.

Keywords Abiotic stress, drought, PGPR, salinity, temperature, PAMs

Introduction

Agriculture is considered to be the most vulnerable sector that is often exposed to the plethora of climate-change. Abrupt change in climatic conditions increases the incidence of abiotic and biotic stresses that become major cause for stagnation of productivity in principal crops (Grover *et al.*, 2010). Amongst abiotic factors, interseasonal climatic variability is a concern, which is usually reflected from year-to-year fluctuations in crop yields. The probability of occurrence of extreme climatic events such as drought, salinity, extreme high/low temperatures, flooding stress, heavy metals stress has increased in the last couple of decades, and farmers lack the management options to sustain the agricultural productivity (Kalra *et al.*, 2013).

When farmers see their agricultural crops declining in yield and production due to abiotic stresses, they often expect a dramatic and magical treatment to make them lush, green, and healthy again so that productivity increases. As a result, they start using chemicals and fertilizers disregarding their future effects. The extensive use of certain synthetic organic chemicals in the past decades has led to a number of long-term environmental problems (Arora *et al.*, 2012).

One of the focuses of the present research involves implication of plant associated microbes (PAMs) including plant growth promoting rhizobacteria (PGPR) to combat the harmful effects of these ecological stresses and enhance plant growth and productivity by direct and indirect mechanisms (Kloepper and Schroth, 1978; Arora *et al.*, 2013). PAMs can play important role in conferring resistance to abiotic stresses. These organisms basically include close residents of rhizosphere, rhizoplane, phyllosphere, phylloplane, endophytes and symbiotic fungi that operate through a variety of mechanisms, like triggering stress response that alleviates stress tolerance and induction of novel genes in plants. The development of stress tolerant crop varieties through genetic engineering and plant breeding is essential but a long drawn and costly process, whereas microbial inoculation to alleviate stresses in plants could be a more cost effective, environmental friendly option which is available in a shorter time frame (Grover *et al.*, 2010). The main aim of the present review is to highlight the impact of climatic variations that cause abiotic stresses and to apprehend the role of microorganisms in helping crops to cope with various abiotic pressures.

Climatic variability govern abiotic stress

Global warming and changes in precipitation patterns, lead to several abiotic stresses such as extremes temperatures, drought, flooding, salinity, metal stress, nutrient stress that are bound to have adverse effects on food production (Pandey *et al.*, 2007; Barrios *et al.*, 2008; Selvakumar *et al.*, 2012). Climate change models have predicted that warmer temperatures and increase in the frequency and duration of

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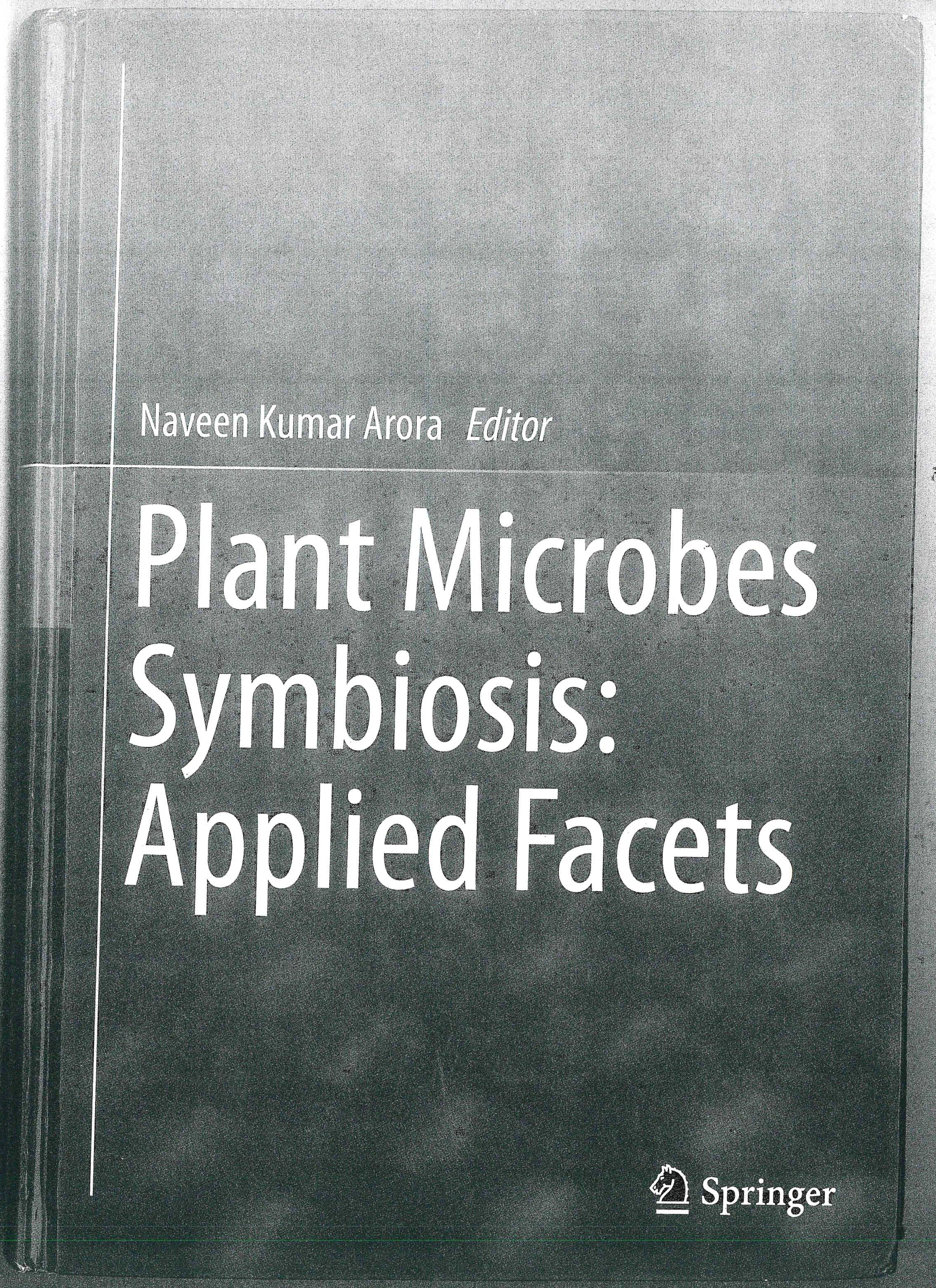
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Biopesticides: Where We Stand?

2

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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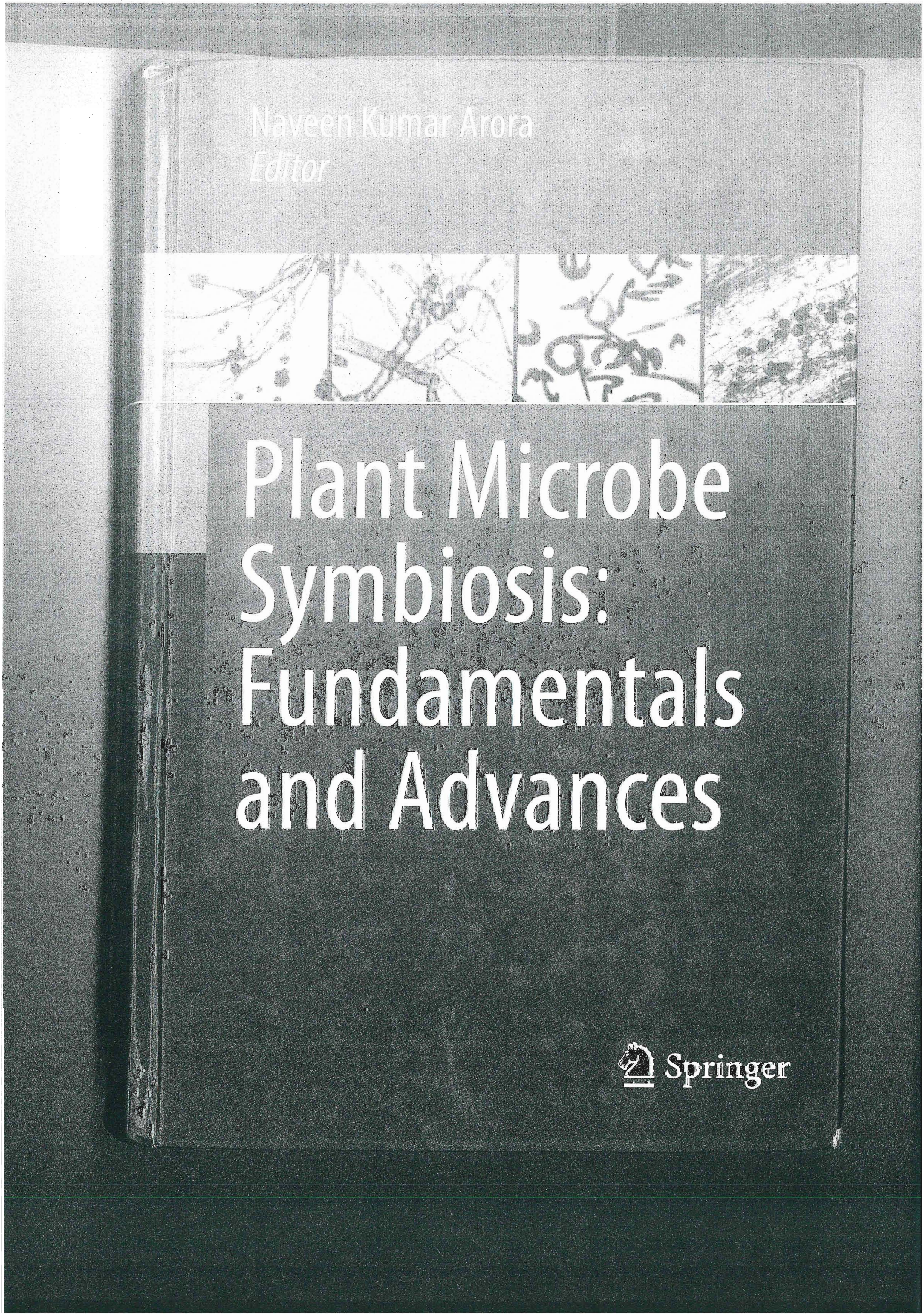
N.K. Arora (ed.), *Plant Microbes Symbiosis: Applied Facets*,
DOI 10.1007/978-81-322-2068-8_2, © Springer India 2015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Chapter 1
Transactions Among Microorganisms and Plant in the Composite Rhizosphere Habitat

Sakshi Tewari and Naveen Kumar Arora

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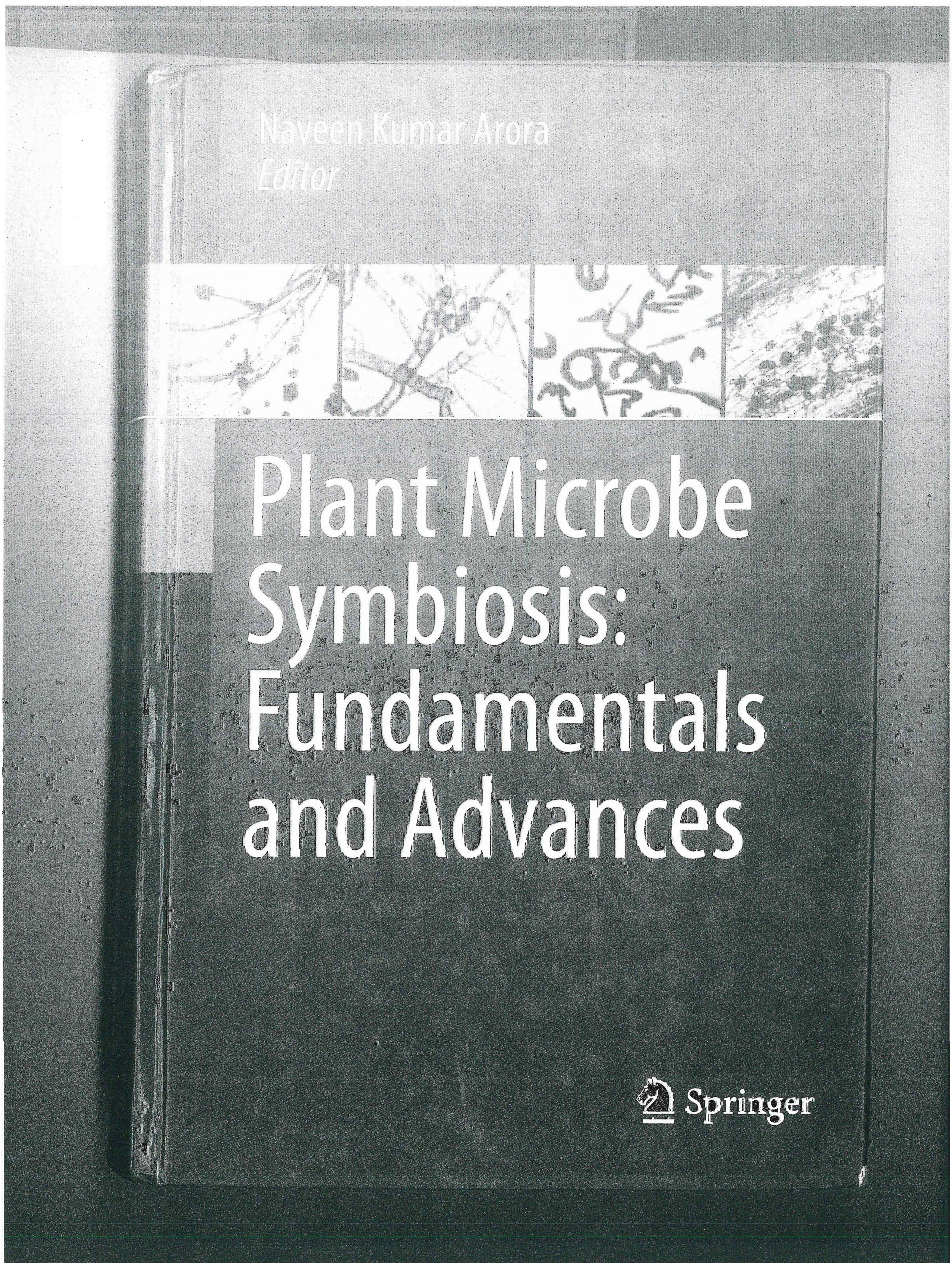
N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_1, © Springer India 2013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Chapter 16
Multifaceted Plant-Associated Microbes and Their Mechanisms Diminish the Concept of Direct and Indirect PGPRs

Naveen Kumar Arora, Sakshi Tewari, and Rachna Singh

Contents

Abstract It is an old saying that when we take from nature, we have to give back also; this give-and-take phenomenon leads to sustainability and is important for growth of a relationship. This is also applicable in plant–microbial world. The association of microbes with plants can be exploited and used to gain the benefits not only for the associated organisms but also for the ecosystem as a whole. When we view it in a holistic way, it is clear that multifaceted and diverse mechanisms of

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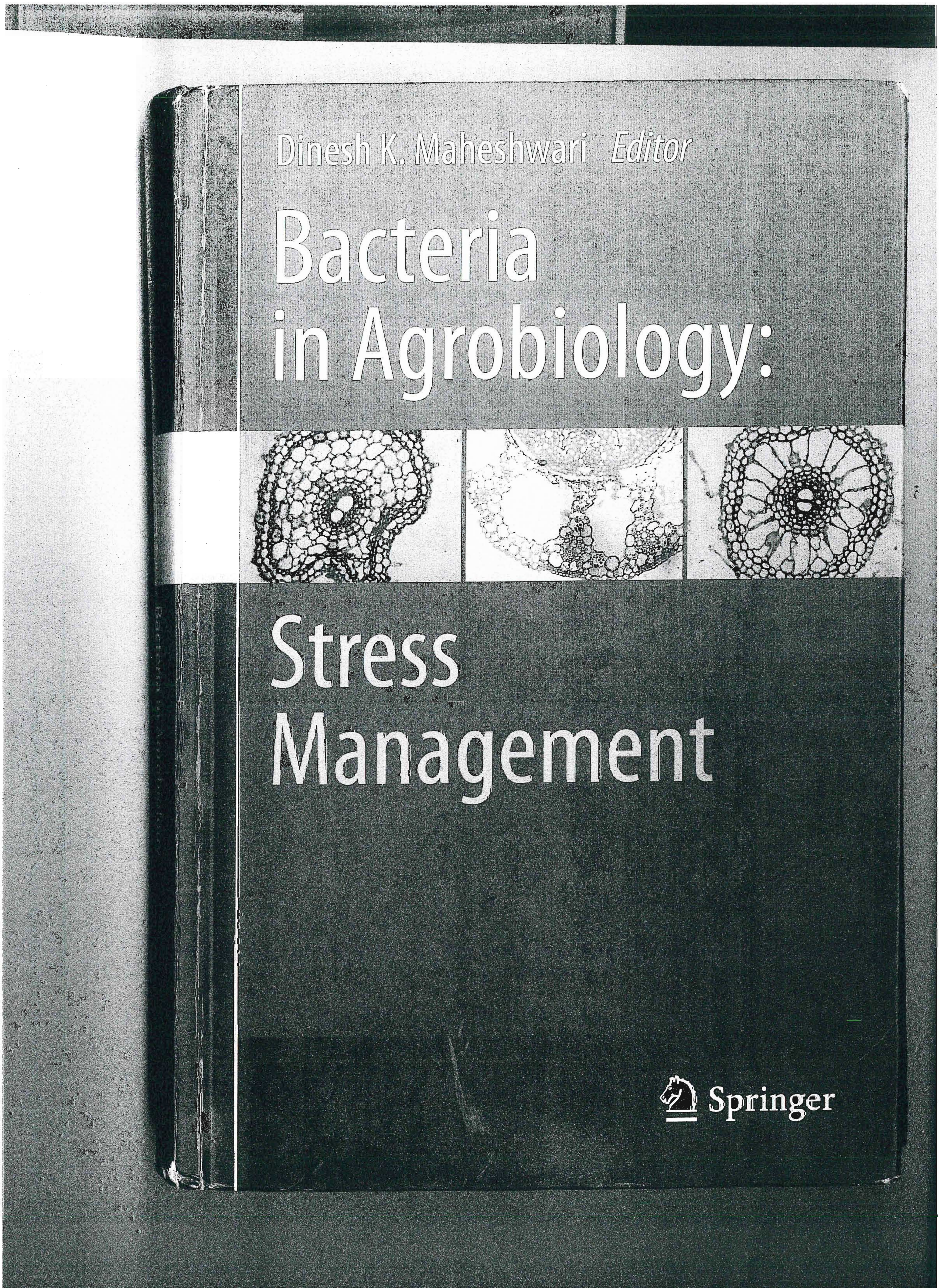
N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_16,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Chapter 12
PGPR for Protection of Plant Health Under Saline Conditions

Naveen K. Arora, Sakshi Tewari, Sachin Singh, Nand Lal, and Dinesh K. Maheshwari

12.1 Introduction

For centuries, agriculture in arid and semiarid environments has faced an increase in soil salinity. Salinity is one of the major abiotic stress factor limiting plant growth and productivity (Khan and Panda 2008). The total salt-affected land worldwide is estimated to be 900 million ha, 6% of the total global land mass (Flowers 2004). According to the Food and Agricultural Organization (FAO), if corrective measures are not taken, salinization of arable land will result in 30% land loss in the next 25 years and up to 50% by the year 2050 (Munns 2002). Salinity prevents plants from taking up water, exposing them to drought stress. These stresses have an adverse effect on plants, hampering their growth and finally production. Soil salinity is defined as the concentration of dissolvable salts extracted from soil by water (Richards 1954). Natural boundaries imposed by soil salinity also limit the caloric and the nutritional potential of agricultural production. These constraints are most acute in the areas devoted to agriculture; therefore, the urgent need of biological agents (biopreparations) is accepted worldwide. Interest in the use of such biopreparations that replenish the soil, add value, and enhance production and yield in saline conditions is the primary recommendation. Plant growth-promoting

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D.K. Maheshwari (ed.), *Bacteria in Agrobiology: Stress Management*,
DOI 10.1007/978-3-642-23465-1_12,