EXPLORATION OF HALOTOLERANT ACTINOMYCETES FROM SALT AFFECTED SOIL AND THEIR UTILIZATION TO AMELIORATE SALINITY STRESS IN WHEAT (*Triticum aestivum* L.)

THESIS
SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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CERTIFICATE

This is to certify that the thesis entitled "**EXPLORATION OF HALOTOLERANT ACTINOMYCETES FROM SALT AFFECTED SOIL AND THEIR UTILIZATION TO AMELIORATE SALINITY STRESS IN WHEAT (*Triticum aestivum* L.)"** submitted by "**Ms. Pooja Shrivastava**" is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

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

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DECLARATION

I, **Pooja Shrivastava**, hereby declare that the thesis work entitled "**Exploration of halotolerant actinomycetes from salt affected soil and their utilization to ameliorate salinity stress in wheat** (***Triticum aestivum*** **L.**)" is my own work carried out under the guidance of **Dr. Rajesh Kumar, Associate Professor, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, (A Central University), Lucknow.** The matter embodied in this thesis work is written by me and has not been submitted to any other university for the fulfilment of the requirement of any other Degree or Diploma.

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(Pooja Shrivastava)

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ABBREVIATIONS

AIA	:	Actinomycetes Isolation Agar
ANOVA	:	Analysis For Variance
AR	:	Analytical Reagent
ARDRA	:	Amplified Ribosomal DNA Restriction Analysis
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base Pair
BSA	:	Bovine Serum Albumin
^oC	:	Degree Celsius
$CaCO_3$	:	Calcium Carbonate
CAS	:	Chrome Azurol S
CMC	:	Carboxy Methyl Cellulose
DAS	:	Days After Sowing
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide Triphosphate
EC	:	Electrical conductivity
g	:	Gram
$g\ L^{-1}$	:	Gram per Litre
h	:	Hour
H_2S	:	Hydrogen Sulfide
HCN	:	Hydrogen Cyanide
$HgCl_2$	:	Mercuric Chloride
HVA	:	Humic Acid Vitamin Agar
IAA	:	Indole Acetic Acid
IGP	:	Indo-Gangetic Plains
ISP	:	International Streptomyces Project
Kb	:	Kilobase
Kg	:	Kilogram
$Kg\ ha^{-1}$		Kilogram per hectare
MEGA	:	Molecular Evolutionary Genetics Analysis
$MgCl_2$	:	Magnesium Chloride

mL	Millilitre
mM	Millimolar
MP	*Macrophomina phaseolina*
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
ng/µL	Nanogram per microliter
No.	Number
NJ	Neighbor Joining
Nm	Nanometer
NTSYS	Numerical Taxonomy and Multivariate Analysis System
OD	Optical Density
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGP	Plant Growth Promotory
pH	Potential of Hydrogen
Pmol	Picomole
PVK	Pikovskaya Agar
RFLP	Restriction Fragment Length Polymorphism
RNase-A	Ribonuclease-A
rpm	Revolutions Per Minute
rDNA	Ribosomal DNA
rRNA	Ribosomonal Ribonucleic Acid
SCA	Starch Casein Agar
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SIM	Sulfide Indole Motility Agar
SPSS	Statistical Package For Social Sciences
SR	*Scleortium rolfsii*
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

USA : United States of America

UV : Ultra-violet

UV-V : Ultra Voilet Visible

µg mg^{-1} : Microgram Per milligram

µM : Micro Molar

V : Volume

w/v : Weight by volume

Chapter 1

INTRODUCTION

Chapter 1

Introduction

Agricultural crops are exposed to a variety of stresses including both biotic and abiotic including change in climate (Saharan and Nehra 2011). Agriculture is considered to be one of the most vulnerable sectors adversely affected by climate change. Climate change could be a mega catastrophe unless the potential calamity is converted into an opportunity for sustainable food security and economic development. Agriculture sector at present is facing a lot of challenges on a global scale, viz., target to produce 70% more food for an additional 2.3 billion people by 2050, while at the same time fighting with poverty and hunger, as well as efficient utilization of scarce natural resources to combat adversities due to climate change (FAO 2009). It is also a fact that productivity of important crops is not increasing in parallel with the ever increasing food demand. Such low productivity in most of the cases is attributed mainly to various abiotic stresses, and thus, curtailing crop losses is a major area of concern in recent times (Shanker and Venkateswarlu 2011). Salinization of soil (abiotic stress) is an emergent and severe problem for worldwide agriculture. Salt accumulation in arable soils is mainly attributed to irrigation water that contains traces of sodium chloride (NaCl) from seawater (Tester and Davenport 2003; Deinlein et al. 2014). Moreover, future climate change-predicted scenarios show the increasing risk of salinization at different latitudes, and therefore, all-out efforts will be required so as to maximally exploit soils under salt stress leading to increased food production (Turral et al. 2011).

Soil salinity

Soil is considered saline if it contains salts in a concentration that is sufficient to interfere with the growth of most crop species. Electrical conductivity is a measure of the ease with which electrical current will pass through water: the greater the salinity, the greater the conductivity; however, the relationship is a function of the specific ions present in the solution and their concentrations. Saline soils have an electrical conductivity of >4 dSm^{-1} (~36 mM NaCl) measured on a saturated soil paste extract at 25°C (US Salinity Laboratory 1969). Depending on the soil texture, the relative water and salt content, and osmotic pressure of the saturation extract can be very different (US Salinity Laboratory, 1969; Bui 2013). Saline soils could be distinguished from those of sodic soils (Na >15%, and with pH >8.5) as these soils do have an electrical conductivity >4 dSm^{-1}, pH <8.5, and Na <15% of total exchangeable cations.

The extent of problem

Salinity has posed a significant challenge to global agriculture, and it is predicted to become severe in the coming decades. Worldwide, ~831 million hectares (mha) of the agricultural land is salt-affected, either due to salinity (397 mha) or due to associated conditions of sodicity (434 mha; FAO 2005; Fahad et al. 2015); such conditions lead to reduced area available for agriculture (reduction rate 1–2% per annum), hitting the hardest in the arid and semi-arid regions (Paul and Lade 2014). According to an estimate, saline soil has adversely affected ~30% of the irrigated and ~6% of total land area in the world (Chaves et al. 2009) with a resultant monetary loss of 12 billion US $ in the form of agricultural produce (Shabala 2013; Fahad et al. 2015).

It has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Jamil et al. 2011; Shrivastava and Kumar 2015). In India, ~7 million hectares of land is covered by saline soil (Patel et al. 2011). This is a dangerous situation, since India supports 18% of the world’s human population and 15% of the global livestock population, but shares only 2.4% of land area. Despite its low proportional land area, India ranks second worldwide in farm output. Agriculture, forestry, and fisheries account for 17% of the gross domestic product and employs about 50% of the total work force of the country. Causes of soil degradation are both natural and human-induced. In arid, semi-arid and sub-humid regions, large areas have been rendered barren due to the development of saline-sodic soils mainly because of poor irrigation and drainage management (Bhattacharyya et al. 2015). Most of the productive agricultural land of India falls in Indo-Gangetic Plains (IGP). There is a perceptible degradation of natural resources in and outside the IGP and a significant stretch of fertile agriculture lands in IGP is unproductive due to the land degradation from salinity and alkalinity (Mandal and Sharma 2006). According to the estimates by the National Bureau of Soil Survey and Land Use Planning, 22.84 m ha in the IGP is degraded (Kumar et al. 1999). Growing salinity hazard, lowering water table (overexploitation of groundwater) and decreased soil fertility are the major concerns.

In northern India, these soils are spread over the states of Uttar Pradesh, Haryana, Punjab, Bihar, and Delhi. In the west, the states of Gujarat, Rajasthan, and Maharashtra have sizable area of salt affected soils. In the central and southern part of the country, the states of Madhya Pradesh, Andhra Pradesh, Karnataka, and Tamil Nadu have extensive areas affected by salty soil. In the east, these are found in the deltaic and coastal parts of West Bengal and Orissa (Singh et al. 2010).

Strategies to mitigate salinity stress in plants

Salinization could be restricted by leaching of salt from root zone, changing of farm management practices and use of salt tolerant plants. Irrigated agriculture can be sustained by employing better irrigation practices, such as adoption of partial root zone drying methodology, and drip or micro-irrigation so as to efficiently utilize water resources. The spread of dry land salinity could be contained by reducing the amount of water passing beyond the roots; this could be achieved by re-introducing deep rooted perennial plants that continue to grow and use water during the seasons that do not support annual crop plants. This may restore the balance between rainfall and water use, thus preventing rising water tables and the movement of salt to the soil surface (Manchanda and Garg 2008). Farming systems can be modified to incorporate perennials in rotation with annual crops (phase farming), in mixed plantings (alley farming, intercropping), or in site-specific plantings (precision farming; Munns et al. 2002). Although the use of these approaches to sustainable soil and crop management can ameliorate yield reduction under salinity stress, but their implementation is often limited because of high cost and non-availability of good quality water. Thus, evolving efficient, low-cost, easily adaptable methods for the abiotic stress management is still a major challenge. Worldwide, extensive research is being carried out, to develop strategies to cope with abiotic stresses through development of salt/drought tolerant varieties, shifting of crop calendars, and efficient resource management practices, etc. (Venkateswarlu and Shanker 2009).

Use of salt-tolerant varieties is an effective strategy to combat the problem of soil salinity. Salt tolerance in crops will also allow the more effective use of poor quality irrigation water. Tolerance will not only be required for ‘‘de-watering’’ species, but also for the annual crops to follow, as salt will be left in the soil when the water table is lowered. To increase the salt-tolerance, there is a need for understanding the mechanisms of salt limitation on plant growth and the mechanism of salt tolerance at the whole-plant, organelle, and molecular levels (Shrivastava and Kumar 2015). When a plant is subjected to abiotic stress, a number of genes are ‘switched on’ resulting in increased levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection against these stresses (Bhatnagar-Mathur et al. 2008). Efforts to improve crop performance by transgenic approach under environmental stresses have yielded much tangible results because the complex fundamental mechanisms of stress tolerance in plant remains to be completely deciphered. Since long, development of salt-tolerant varieties has been a major objective of plant breeding programs in order to maintain crop productivity in semiarid and saline lands. Although, several salt-tolerant varieties have been developed and released, the overall progress of traditional breeding has been slow and has not been much successful (Schubert et al. 2009; Dodd and Perez-Alfocea 2012).

Though a lot of approaches have been applied for development of salt tolerant varieties through transgenic routes but complete success is yet to be achieved so far. The assessment of salt tolerance in transgenic experiments has been mostly carried out using a limited number of seedlings or mature plants under standard laboratory conditions, which are not easy to simulate or match to the actual high-salinity soil conditions (e.g., alkaline soil pH, high diurnal temperatures, low humidity, and presence of other sodic salts and elevated concentrations of selenium and/or boron). This is the reason; plants evaluated under laboratory conditions show a little correlation to tolerance in the field. In addition, the variable salt levels in different field conditions also aggravate the problem of effective evaluation (Richards 1983).

Nevertheless, there have been many successes in developing stress-tolerant transgenics in model plants like tobacco, Arabidopsis and rice (Grover et al., 2003), which necessitates to test such advances in economically important crop species.

Microbes as effective candidates for amelioration of salt stress in plants

Several strategies including plant genetic engineering approaches have been developed in order to decrease the toxic effects caused due to high salinity on plant growth (Wang et al. 2003), and recently the use of plant growth-promoting bacteria (PGPB) (Dimkpa et al. 2009) have been exploited. The identification and exploitation of soil microorganisms that interact with plants by alleviating stress opens new avenues for pyramiding strategies against salinity; this also allows discovering and studying new mechanisms involved in stress tolerance (Dodd and Perez-Alfocea 2012). Soil aggregate stability is one of the most important properties controlling the growth of plants in semi-arid environments. Therefore, the improvement of soil structural stability is of great significance and the contribution of microbial populations, either as free-living or rhizospheric organisms, and their activities to soil aggregate stability is proved to be highly beneficial (Jastrow and Miller 1991). The role of microorganisms in plant growth promotion, nutrient management and disease control is well- known and established. Such beneficial microorganisms colonize the rhizosphere/endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms (Nia et al. 2012; Ramadoss et al. 2013). Generally, plant growth promoting microbes work directly, by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick 2012). The occurrence of several of beneficial rhizobacteria like *Rhizobium*, *Streptomyces*, *Bradyrhizobium, Azotobacter, Azospirillum, Pseudomonas*, *Bacillus*, etc. has been reported from stressed environments like desert ecosystems, acid soils, saline and alkaline areas and highly eroded hill slopes (Selvakumar et al. 2009; Upadhyay et al. 2009; Palaniyandi et al. 2014). Apart from developing mechanisms for their own stress tolerance, plant growth promoting rhizobacteria (PGPR) can also impart some degree of tolerance to plants towards abiotic stresses including salinity. The term Induced Systemic Tolerance (IST) has been proposed for PGPR-induced physical and chemical changes that result in enhanced tolerance to abiotic stress (Shrivastava and Kumar, 2015). Interaction of PGPR with several crops in saline conditions reduces the extent of poor growth and thus helps plant survive and show improved performance in adverse conditions (Dimkpa et al. 2009). Reports also indicate that bacteria isolated from a saline environment are more likely to survive inhibitory salt concentrations than their counterparts from non-saline habitats (Hua et al. 1982; Siddikee et al. 2011). Salinity tolerance and improved plant performance due to co-inoculation with stress tolerant beneficial microbes is reported in several plants *viz.* tomato (Mayak et al. 2004), maize (Nadeem et al. 2007), rice (Jha et al. 2011), wheat (Nadeem et al. 2013), barley and oats (Chang et al. 2014) etc.

Halotolerant actinomycetes in salinity tolerance of plants

Actinomycetes are precious prokaryotes both in terms of economy and research which have provided an array of important bioactive compounds (Solanki et al. 2008). Several ecological studies on actinomycetes have shown that these microorganisms are widespread in nature and may also occur in extreme environments. Thus, groups of acidophilic and alkaliphilic, psychrophilic and thermophilic, halophilic and haloalkaliphilic and xerophilic actinomycetes have been reported (Al-Tai et al. 1994; Jiang and Xu 1993; Selianin et al. 2005). Despite these reports, little is known about the diversity of actinomycetes from saline environments (Meklat et al. 2011). They are generally involved in the decomposition of organic materials in soil. Members of the genus *Frankia* fix nitrogen in nodules of non-leguminous plants. Actinomycetes have been used in the biological control of plant pathogens, and a few are known as plant pathogens (Heuer et al. 1997). Plant growth-promoting actinomycetes can prevent the deleterious effects of stressors from the environment by accumulating compatible solutes in their cytosol, which protects them from cytoplasmic dehydration and salt stress (Yancey et al. 1982). Majority of bacterium accumulates sugars (e.g. trehalose), polyols (e.g. proline, betaine and ectoine), when exposed to osmotic shock. Gram-positive bacteria including *Streptomyces* species have been reported to accumulate the neutral amino acids like proline (Killham and Firestone, 1984) in response to increasing salt stress, which helps them to maintain their existence in adverse condition. The phytohormone (auxin), siderophore, capability to solubilise phosphate and compatible solute produced by halotolerant actinomycetes plays an important role in alleviating salt stress in plants and these organisms should therefore be considered as a seed dressing in field trials to improve growth and yield of crop plants in farms with high soil salinity (Egamberdieva 2012; Sadeghi et al. 2012).

Importance of proposed research

Excess accumulation of salts in soil is a major ecological and agronomical problem worldwide that affects almost all important food crops including the most important staple crops like rice and wheat. Although the important physiological insights about the mechanism of salt tolerance in crop plants have been gained, but the transfer of such knowledge in to the practical solution is yet to become a reality. Developing salt tolerant crop varieties has been a much desired scientific goal, but with little success. To date, a few major determinant genetic traits of salt tolerance have been identified. An alternative strategy to alleviate salinity stress in crops may be to introduce halotolerant microbes that play-around with rhizosphere and enhance crop growth. In fact, recent studies have demonstrated that adaptation of plants to local environment is driven by genetic association with rhizospheric microbes. Therefore, halotolerant actinomycetes were isolated from salt-affected region of Indo-gangetic plain of Uttar Pradesh, India, since, this is one of the most productive and fluvial plains for excellent wheat production.

A lot of research is being carried out for sustainable approaches for addressing the salinity issue using microbes but most of them are concentrated on bacteria and arbuscular mycorrhizal fungus (AMF). Very few reports are there on the role of halotolerant actinomycetes in salinity stress amelioration in crop (wheat) in this region, so the present study was undertaken with the objectives given in the next section below. In this study actinomycetes were chosen because: (i) they are important microbes present in soil with *Streptomyces* being one of the predominant soil genera, (ii) they are reported from moderately saline to extremely saline habitats, (iii) capable of producing plant growth promotory compounds *viz.* IAA, siderophore, ammonia and solubilise phosphate, and (iv) are well-known for the antimicrobial metabolites. So the halotolerant actinomycetes were selected to study their plant growth promotory properties in saline conditions along with efficiency to produce proline the compatible solute. Further, efficiency of the most potent isolates were evaluated for enhancement of wheat growth and yield under stressed conditions.

Major objectives

To address the gaps in knowledge regarding the halotolerance capability, molecular diversity, plant growth promotory attributes, osmolyte (proline) synthesis and enhancement of wheat growth in saline soil by salt tolerant actinomycetes from salt-affected region of IGP, Uttar Pradesh, India, this research work was undertaken with following major objectives:

OBJECTIVES

1. Isolation and screening of actinomycetes from salt affected regions.
2. Characterization of the isolates for their salt tolerance capacity and plant growth promoting attributes.
3. Biochemical and molecular characterization of the potent isolates.
4. Estimation of proline (osmolyte) production by salt tolerant actinomycetes.
5. Evaluation of the promising isolates by pot experiments for wheat growth promotion under saline and non-saline conditions.

Chapter 2

REVIEW OF LITERATURE

Chapter 2

Review of Literature

2.1 Salt-affected soil and Salinity: Problem and global scenario

In the era of climate change, depleting soil fertility and reducing ground water table has increased the pressure on land resources leading to different types of degradation *viz.* soil salinization (Behera et al. 2015). Salt-affected soil can be defined as soil with high levels of dissolved salts and/or high concentrations of adsorbed sodium ions in the soil matrix (Qadir et al. 2000). They are classified into three classes based on salinity and sodicity values, represented by electrical conductivity (ECe) and sodium adsorption ratio (SAR) or exchangeable sodium percentage (ESP): saline, saline-sodic, and sodic soils (Jesus et al. 2015). Saline-sodic and sodic soils are generally bracketed together because similar amelioration practices are used for these soils (Qadir et al. 2000). Salt-affected soils are identified by excessive levels of water-soluble salts, especially sodium chloride (NaCl) (Tanji, 2002). Other major cations in salt-affected soils are sodium (Na^+), calcium (Ca^+), magnesium (Mg^{2+}), and, to a lesser extent, potassium (K^+). The major anions are chloride (Cl^-), sulphate (SO_4^{2-}), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and nitrate (NO_3^-). Excess of salts in a soil can bring drastic and complex changes in some of the soils physical and chemical properties rendering its environment unsuitable for growth of most crops.

Salinization is recognized as the potential threat to environmental resources in many countries and affects almost one billion hectares of land worldwide (Munns and Tester 2008; FAO Land and Nutrition Management Service 2008). Salt-affected soil exists mostly under arid and semiarid regions and is one of the major causes of declining agricultural productivity. In fact, salt-affected soils in arid and semi-arid regions of Asia, Africa, and South America cause considerable agronomic problems. In Asia alone, 21.5 million ha of land area is thought to be salt affected (of which 12 million ha is due to saline conditions and the remaining 9.5 million ha is due to alkaline/sodic conditions) (Sahi et al. 2006). Salinity affects about 20% of all irrigated agricultural fields and over 7% of the world land surface (Szabolcs 1994), by reducing the ability of crops to take up water and due to ion toxicity. The physiological consequence of this is the loss of the crops by inhibition of seed germination, seedling growth, flowering and fruit set (Sairam and Tyagi 2004). The United Nations, Food and Agriculture Organization (FAO) estimates show that Africa, Asia, Australia, Europe, Latin America, Near East and North America could be the worst affected areas due to salty soils (Koohafkan 2012) as depicted in Table 2.1. In addition, the increased rate (10% per annum) of expansion of salinized areas due to low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices aggravate the whole problem.

Table 2.1 Regional distribution of salt affected soil in all continents.

Region	**Total area (Mha)**	**Saline soils**		**Sodic soils**	
		Mha	**%**	**Mha**	**%**
Africa	1,899	39	2.0	34	1.8
Asia, the Pacific and Australia	3,107	195	6.3	249	8.0
Europe	2,011	7	0.3	73	3.6
Latin America	2,039	61	3.0	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
Total	**12,781**	**397**	**3.1**	**434**	**3.4**

Source: Paul and Lade 2014

Major factors increasing salinity include irrigation of cultivated lands with saline water, poor cultural practices, and low precipitation. Indeed salt stress problems in field crops are getting accentuated with the increasing irrigation networks (Sahi et al. 2006). About 77 million hectares of global salt affected lands have been salinized as a result of human activities, with 58% of these concentrated in irrigated lands. Generally speaking, over 40% of irrigated lands in the world are subject to different degrees of salinity (Dakheel, 2005). As a consequence of increasing population pressure, more arid land will be put into agricultural production in future, which means more salinization danger associated with irrigations

(Alhammadi and Kurup 2012). Almost 300 million hectares in the world are irrigated. Irrigated agriculture consumes about 90% of the total water withdrawal to produce 36% of the global food (Rengasamy 2006; ICID 2009). It has been estimated that inappropriate irrigation/drainage practices affect approximately fifty percent of the global irrigated areas, with an annual increase of up to 500,000 ha. These facts represent a serious threat to sustainable food production and to our natural resources (Ondrasek et al. 2009, Egamberdieva and Lugtenberg 2014).

Economical global annual costs by loss in crop production caused by salt-induced land degradation were estimated to be US$ 27.3 billion (Qadir et al. 2014). Indeed at a global scale, Ghassemi et al. (1995) reported annual income losses from salt-affected irrigated areas at around $12 billion, based mainly on crop yield losses. They reported 45.4 million ha irrigated area as salt-affected, i.e. 20% of the global irrigated area in 1990 (227 million ha). Based on this estimate, the annual cost of salt-induced land degradation was approximately $264 ha^{-1} in 1990. Since this assessment, there has been an increase in the extent of irrigated area vis-à-vis salt-induced land degradation. In 2002, the global irrigated area was estimated at 277 million ha. Considering 20% of this area affected by salts (55.4 million ha) and the same cost of land degradation valued in 1990 ($264 ha–1), the annual economic losses in 2002 were $14.6 billion. Adjusting for $ inflation during the 1990-2002 period, the cost of salt-induced land degradation in 2002 was $345 ha^{-1} with global losses of $19.1 billion. Considering current extent of global irrigated area as 310 million ha (FAO-AQUASTAT, 2013) and 20% of this area as salt-affected (62 million ha) and the inflation-adjusted cost of salt-induced land degradation in 2013 as $441 ha^{-1}, a simple benefit transfer suggests that the current annual economic losses could be $27.3 billion.

2.2 Classification of salt affected soil

In a broader perspective, salt-affected soil is a soil that has been adversely modified for the growth of most crop plants by the presence of soluble salts, with or without high amounts of exchangeable Na (SSSA glossary, https://www.soils.org/publications/soilsglossary#). Salt-affected soils based on their chemistry, morphology, pH, total soluble salt content and the exchangeable sodium percentage

(ESP) or sodium adsorption ratio (SAR) are categorised mainly in to three classes, viz., saline, sodic and saline-sodic soils (United States Salinity Laboratory 1969, Alhammadi and Kurup 2012).

i) Saline soils: These soils have a saturated paste electrical conductivity (ECe) of 4 dS m^{-1} or more and SAR of <12 (ESP <15%). The pH of these soils, which were formerly referred to as white alkali soils, is less than 8.5. Because of a high salt content, these soils reduce water uptake by plants, and increase ion toxicity to the plant tissues. Further, ion imbalances may also occur in some soils.

ii) Sodic soils: These soils have an ECe of less than 4 dS m^{-1}, and SAR of 12 or more (ESP ≥15%), and their pH generally exceeds 8.5. Exchangeable sodium percentage of 15 indicates that Na^+ occupies more than 15% of the cation exchange capacity of soil. Previously, such soils were known as black alkali because of the dispersed black organic matter coatings on the soil surface. The most important problems for these soils are poor structure due to breakdown of structural units, ion toxicity (mainly Na^+ and Cl^-), and ion imbalances, especially deficiencies in Ca^{+2}, Mg^{+2}, and K^+.

iii) Saline-sodic soils: These soils have ECe $\geq$ 4 dS m^{-1}, SAR $\geq$ 12, and their pH is less than 8.5. They have similar problems to saline soils, especially reduced water uptake due to high soil osmotic potential.

2.3. Status of salinity affected soil in India with reference to Indo-gangetic plains

Like the problem of salt-affected soils afflicting many other countries with arid and semi-arid climates, these lands occupy about 8.6 M ha in India, out of which 3.5 M ha are in canal commands, commonly referred to as man-made or wet deserts (Minhas and Sharma 2003). In India, the problem of salinity and alkalinity increases every year as a result of secondary salinisation. The salt affected soils are distributed in fourteen states and the Andaman and Nicobar Islands. An extensive area is occurring in the Indo-Gangetic Plain (IGP) of Uttar Pradesh, Bihar, Haryana, Punjab and West Bengal. It also occupies significant area in the arid and semiarid regions of Gujarat, Rajasthan, Madhya Pradesh and Maharashtra and the peninsular region of Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh and Orissa. Sizeable area is distributed in east and west coast of Andhra Pradesh, Orissa, Tamil Nadu, Kerala, Karnataka, Maharashtra (Mandal et al. 2010). The state wise extent and distribution of salt affected soil in India is shown in Table 2.2.

Table 2.2. State-wise extent and distribution of salt affected soils in India

Sl. No.	State	Saline soils (ha)	Sodic soils (ha)	Total (ha)
1	Andhra Pradesh	77598	196609	274207
2	Andaman and Nicobar	77000	0	77000
3	Bihar	47301	105852	153153
4	Gujarat	1680570	541430	2222000
5	Haryana	49157	183399	232556
6	Karnataka	1893	148136	150029
7	Kerala	20000	0	20000
8	Maharashtra	184089	422670	606759
9	Madhya Pradesh	0	139720	139720
10	Orissa	147138	0	147138
11	Punjab	0	151717	151717
12	Rajasthan	195571	179371	374942
13	Tamil Nadu	13231	354784	368015
14	Uttar Pradesh	21989	1346971	1368960
15	West Bengal	441272	0	441272
	Total	**2956809**	**3770659**	**6727468**

Source: Mandal et al. (2010)

Indo-Gangetic Plain (IGP) of India that stretches from the foothills of Himalayas near the Punjab State to the Gangetic delta in West Bengal State is known for highly fertile soil and favourable climatic condition for highest production of rice-wheat. Appearance of soil salinity in large areas of IGP poses a major concern due to loss of productivity. The salt affected soil map of India (NRSA 1997) shows vast areas of salt affected soils distributed along the Gangetic Plain covering the States of Haryana, Punjab, Uttar Pradesh, Bihar and West Bengal (Fig. 2.1). Almost 2.8

million hectares of salt-affected soil are present within the Indo-Gangetic alluvial plain occupying parts of Punjab, Haryana, Uttar Pradesh, Delhi, Bihar and Rajasthan (Abrol et al. 1971). In IGP, salt affected soils occupy a total area of 31,10,717 ha, of which 57% lies in Uttar Pradesh, 19% in West Bengal, 12% in Bihar, 7.3% in Haryana and 4.6% in Punjab (Mandal and Sharma, 2006).

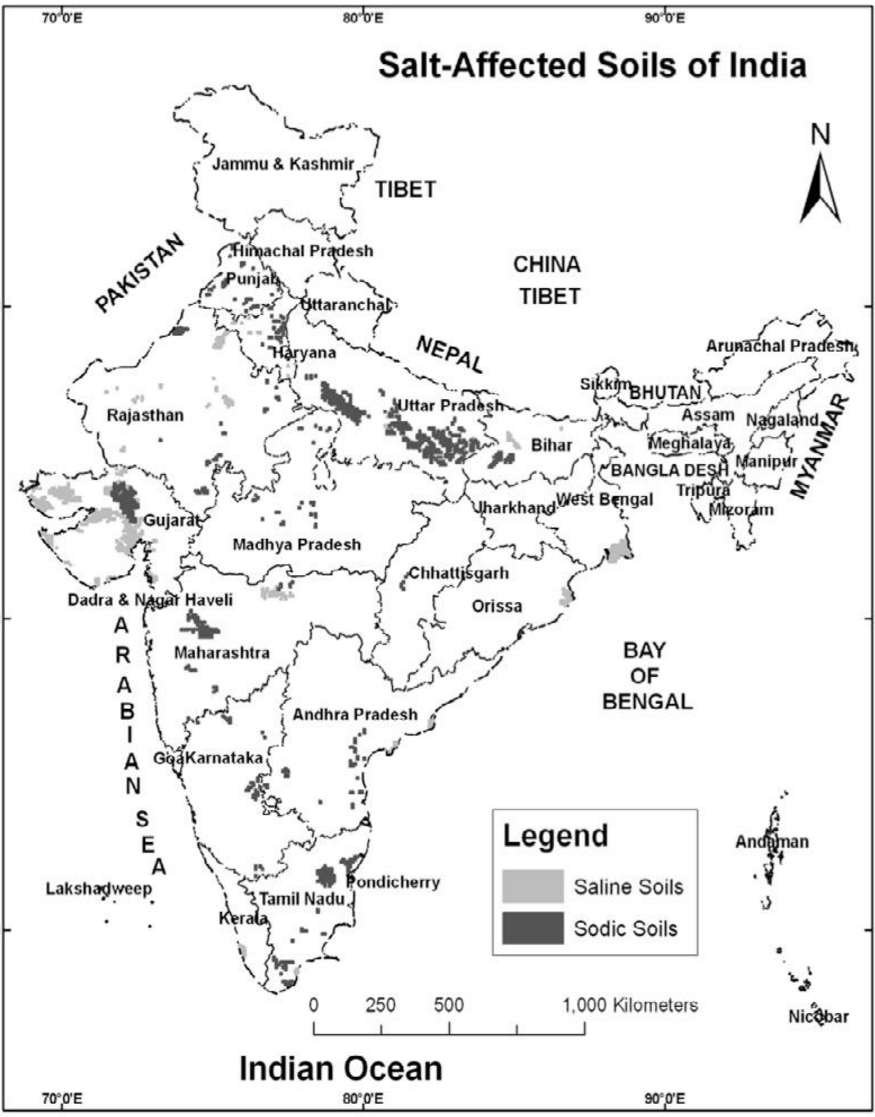

Fig 2.1. Distribution of salt-affected soils in India.

For instance, 109 districts in IGP experience the problem of salinity. Mandal and Sharma (2006) estimated 3.09 mha areas in IGP represents salt affected soils and groundwater surveys have shown that about 41–84% of the well water in different states of IGP is brackish, being the cause of detoriation of soil health.

The potential yields of rice–wheat system in the IGP are very high (ranging from 12.0 to 19.5 t ha^{-1}) due to the favourable climatic factors (Aggarwal et al. 2000). Some progressive farmers in north-western India harvest almost 16 t ha^{-1} from the rice–wheat cropping system, indicating a small 'yield gap'. Nevertheless, this region has also been showing stagnation in yield for some time (Dawe et al. 2000). In contrast, yields in the eastern IGP are low (i.e., showing a large 'yield gap') and are also unstable. While agronomy, plant breeding and policy options could increase the yield potential in the western IGP still further, information on how to increase yield stability and reduce the yield gap in the eastern IGP is urgently needed; given the large yield gap, this region could be the future source of food security for the whole of South Asia. Large parts of the IGP are currently food insecure (Swaminathan, 2002). The role of microbiology and biotechnology may be critical in alleviating abiotic and biotic constraints (Aggarwal et al. 2004) in these regions.

2.4. Impact of soil salinity on growth and development of plants

Soil salinity adversely affects plant growth, crop production, soil and water quality and eventually results in soil erosion and land degradation (Zhu, 2001). Development of soil salinity is a dynamic process with severe consequences for the soil, hydrological, climatic, geochemical, agricultural, social and economic aspects (Allbed and Kumar 2013). Agricultural crops exhibit a spectrum of responses under salt stress. Salinity not only decreases the agricultural production of most crops, but also, affects soil physic-chemical properties, and ecological balance of the area. The impacts of salinity include—low agricultural productivity, low economic returns and soil erosions, (Hu and Schmidhalter 2002). Salinity effects are the results of complex interactions among morphological, physiological, and biochemical processes including seed germination, plant growth, and water and nutrient uptake (Akbarimoghaddam et al. 2011; Shrivastava and Kumar 2015). Salinization of soil negatively affects developmental stages in plants and induces premature leaf senescence with corresponding detrimental effect on overall crop yield (Flowers et al. 2010). Salt-stressed plants face at least three major constraints, viz., (i) water deficiency, (ii) ion toxicity, and (iii) ion imbalance. Reduced water availability due to the decrease in the soil osmotic potential affects the most sensitive species, which are unable to regulate their water potential with respect to the soil, resulting in the loss of cell turgor. In addition, excess ions, particularly, Na^+ and Cl^- are extremely toxic for most of the crop plants that negatively affects enzyme activity and membrane stability resulting to enhanced reactive oxygen species (ROS) activity (Cabot et al. 2014). Compared to Chloride (Cl^-), Sodium (Na^+) is more toxic to plants, as it causes higher ion-specific damage resulting to a range of disorders related to enzyme activation and protein synthesis (Tester and Davenport 2003). Conversely, an excess of Cl^- accumulation inhibits photosynthesis. With the onset and development of salt stress within a plant, all the major biological processes, *viz.*, photosynthesis, protein synthesis, and energy and lipid metabolism are adversely affected (Parida and Das 2005). Due to the osmotic stress and partial stomata closure, the photosynthetic capacity is reduced (Meloni et al. 2003). Plants may also suffer from membrane destabilisation and a general nutrient imbalance (Hasegawa et al. 2000). In many crop plants, plant growth and yield is affected by salinity in varying degrees depending upon the EC of the soil (Table 2.3). As the salinity increases, growth decreases until plants become chlorotic and eventually die. Based on EC, soil salinity could be categorised in to five classes (Table 2.3; Paul and Lade 2014).

Table 2.3. Effects of varying levels of salinity on crop plants (adopted from Paul and Lade 2014)

Soil salinity class	Electrical conductivity of the saturation extract (dS/m)	Effect on crop plants
Non-saline	0-2	Salinity effects are negligible
Slightly saline	2-4	Yield of sensitive crops may be restricted
Moderately saline	4-8	Yield of many crops restricted
Strongly saline	8-16	Only tolerant crops yield satisfactorily
Very strongly saline	>16	Only a few very tolerant crops yield satisfactorily

Under saline conditions, osmotic pressure in the rhizosphere exceeds that in root cells influencing water and nutrient uptake. Almost all micro- and macro-nutrient contents decrease in the root and shoot with increasing NaCl concentrations in the growth medium (Paul and Lade 2014). The salinity also gives rise to numerous secondary stresses, *viz.*, oxidative stress characterised by accumulation of Reactive Oxygen Species (ROS), biomembrane instability, proteins and nucleic acid degradation, and enzyme inactivation, etc. (Porcel et al. 2012). Unfortunately, most crop plants are on the glycophyte side of the salt tolerance spectrum and main cereal crops such as wheat, corn and rice as well as leguminous species are quite sensitive to saline conditions (Cabot et al. 2014).

Wheat is one of the important and extensively grown staple crops of the world with China as its largest producer followed by India. But in the last few years it has suffered from severe salt stress resulting in low grain yield, poor quality (Zhang et al. 2013, 2016), and yield losses of ~65% in moderately saline soils (Shafi et al. 2010). Soil salinity significantly reduces plant nutrient uptake, particularly, phosphate which gets precipitated with calcium (Ca) ions in saline soils (Grattan and Grieve 1998, Upadhyay et al. 2012). Wheat is a main cereal crop that provides protein and carbohydrate for many people, thus requirement for higher quality wheat is very important. Researchers have focused on the effect of salinity on wheat grain yield and found that reduction in productivity of wheat is a major detrimental result due to salinity. The adverse effects of salinity on plant development are more profound during the reproductive phase. Wheat plants stressed at 100–175 mM NaCl showed a significant reduction in spikelets per spike, delayed spike emergence and reduced fertility, which result in poor grain yields. However, Na^+ and Cl^- concentrations in the shoot apex of these wheat plants were below 50 and 30 mM, respectively, which is too low to limit metabolic reactions (Munns and Rawson, 1999). Hence, the adverse effects of salinity may be attributed to the salt-stress effect on the cell cycle and differentiation. Salinity arrests the cell cycle transiently by reducing the expression and activity of cyclins and cyclin-dependent kinases that results in fewer cells in the meristem, thus limiting growth. Soil salinity has adverse effects on number of grains plant^{-1} and grain yield plant^{-1} thus;

improving its productivity under saline conditions is a major area of concern to ensure food security (Talaat and Shawky 2014).

2.5. Metabolomic effect of salinity on crop plants: Accumulation of compatible solutes with special reference to proline

Upon exposure to osmotic stress as a result of low temperature, drought and high salinity, plants accumulate a range of osmolytes with the primary function of turgor maintenance. The solutes accumulated vary among species and include sugars (i.e., sucrose, glucose, fructose and trehalose), polyols, betaines and amino acids, such as proline (Smirnoff 1998; Shulaev et al. 2008). Many compounds are known to play a role as osmoprotectants, acting as low molecular weight chaperones, stabilising the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging the reactive oxygen species (ROS). Examples of these molecules are glycine betaine, proline and mannitol (Chen & Murata 2008; Szabados and Savourè 2010). Other compounds act as chelating agents (sequestering toxic metals and ions), redesigners of lipids (optimising the structure and fluidity in membranes), energy sources and/or signalling molecules (Valluru and Van den Ende 2008; Alcázar et al. 2010). For some compounds, such as proline and glycine betaine, the exogenous application of the molecule or the enhancement of their biosynthesis through the ectopic expression of a rate-limiting gene has resulted in a stress tolerance improvement (Chen & Murata 2008; Kishor et al. 1995).

Among the best known compatible solutes, proline and glycine betaine (GB) have been reported to increase greatly under salt and drought stresses (Munns 2002; Sakamoto & Murata 2002) and constitute the major metabolites found in durum wheat under salt stress, as in other Poaceae’s (Sairam & Tyagi, 2004; Ashraf & Foolad 2007). In many halophytes, proline and/or glycine betaine concentrations in leaves contribute to the osmotic pressure in the cell as a whole (Flowers et al. 1977). In glycophytes, their concentrations are much lower but if partitioned exclusively to the cytoplasm, they could generate a significant osmotic pressure and then balance the vacuolar osmotic potential. In durum wheat seedlings, proline can contribute for more than 39% of the osmotic adjustment in the cytoplasmic compartment of old leaves, while the contribution of GB can account for up to 16% of the osmotic balance in younger tissues, independently of nitrogen nutrition, unlike proline (Carillo et al. 2008).

Proline is a proteinogenic amino acid with an exceptional conformational rigidity, essential for primary metabolism, which normally accumulates in large quantities in response to drought or salinity stress (Ashraf and Foolad 2007). Its accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment (Carillo et al. 2011). In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures (e.g. membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. It may also function as a protein compatible hydrotrope (Srinivas and Balasubramanian 1995), alleviating cytoplasmic acidosis, and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism (Carillo et al. 2011). Also, rapid breakdown of proline upon relief of stress may provide sufficient reducing agents that support mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress induced damages (Carillo et al. 2008). Furthermore, proline is known to induce expression of salt stress responsive genes, which possess proline responsive elements (e.g. PRE, ACTCAT) in their promoters (Ashraf and Foolad 2007; Chinnusamy et al. 2005). Under abiotic stress conditions, increased proline biosynthesis was observed for various plant species inoculated with different PGPR (Barka et al. 2006; Kohler et al. 2009; Jha et al. 2010; Sandhya et al. 2010; Vardharajula et al. 2011). The synthesis of proline as well as other compatible solutes require energy cost (41 moles of ATP) and occur at the expense of plant growth, but may allow the plant to survive and recover from the presence of high external salt concentration (Munns & Tester 2008).

2.6. Mitigation of salt stress in crops: breeding and genetic engineering approach

The success of plant biotechnology programs has helped the world’s food supply keep pace with the increasing rate of population growth (Morrissey et al. 2004). Novel crop varietals with superior yields, as well as increased tolerance to biotic and abiotic stresses, have been continuously produced for decades through conventional plant breeding programs, and more recently through genetic engineering (Atkinson and Urwin 2012). Despite the undeniable success of these past efforts and their continued applicability to stress-tolerance in crop species, each of these methods has its drawbacks, which should be fully considered. Development of salt-tolerant crops has been a major objective of plant breeding programs for decades in order to maintain crop productivity in semiarid and saline lands. Although, several salt-tolerant varieties have been released, the overall progress of traditional breeding has been slow and has not been successful as only few major determinant genetic traits of salt tolerance have been identified (Schubert et al. 2009; Dodd and Perez-Alfocea 2012). Plant breeding is highly time consuming, as well as labour and cost intensive (Ashraf 2010; Eisenstein 2013). Additionally, in the quest for the improvement of a particular trait, such as drought/salinity tolerance, certain (often unknown) desirable traits can be unintentionally lost from the host's gene pool during conventional breeding (Philippot et al. 2013). Perhaps the largest drawback, however, is that plant breeding only confers benefit to a single host species, and this benefit is often not easily transferable to other crop systems, as the genetic components responsible for the improvements frequently remain unidentified.

To avoid the time and labour costs associated with conventional breeding, some researchers have turned to generation of transgenic lines for producing varietals with improved plant growth regulators, antioxidants, organic osmolytes or other factors capable of increasing drought tolerance (Eisenstein 2013). Unfortunately, the vast majority of these are developed and tested in the greenhouse, rather than in the field and claims made regarding their performance are often inflated compared to actual results in agricultural settings, due to the large array of abiotic and biotic factors left out of the initial experiments (Ashraf 2010). Additionally, these transgenic crops often must pass rigorous food and environmental safety regulations and trials before becoming marketable, which add additional time to the product development process (Eisenstein 2013). Furthermore, release of a transgenic product into the marketplace does not guarantee its success, as public response to use of genetically modified crops varies considerably from country to country (Fedoroff et al. 2010).

2.7 Plant growth promoting microbes: an eco-friendly sustainable approach for salinity stress management

In recent years, plant-associated microbial communities have received considerable attention for their ability to confer many of the same benefits to crop productivity and stress resistance as have been achieved through plant breeding programs (Mayak et al. 2004; Tank and Saraf 2010; Marasco et al. 2012). It is now well recognized that all plants, and nearly all tissues within the plant, are inhabited by a variety of microorganisms (Partida-Martínez and Heil 2011; Berg et al. 2013), many of which offer benefits to the host, improving nutrient uptake, preventing pathogen attack, and increasing plant growth under adverse environmental conditions (Yang et al. 2009; Turner et al. 2013). In return, these microorganisms receive shelter from the surrounding environment and access to a carbon-rich food supply. The most well-studied of these symbionts include the mycorrhizal fungi, which enhance nutrients uptake (Bonfante and Anca 2009) and root-nodulating bacteria, which fix nitrogen from the surrounding soil (Lugtenberg and Kamilova 2009), but many other novel plant growth-promoting microorganisms (PGPM) continue to be identified each year. These organisms confer stress resistance via diverse mechanisms as reviewed in various literature (Lugtenberg and Kamilova 2009; Yang et al. 2009; Grover et al. 2010; Nadeem et al. 2014). Importantly, efforts are being made to harness these naturally occurring, soil-derived beneficial microbes for large-scale improvement of crop performance in agriculture (Nadeem et al. 2014).

Compared with methods for directly engineering stress tolerance into the host described above, symbiont-based approaches to improving stress tolerance offer some clear advantages (Figure 2.2). First, microbial symbionts are frequently capable of conferring stress tolerance to a wide variety of diverse plant hosts, and many PGPM can confer benefits to both monocots and dicot crop species (Timmusk and Wagner 1999; Redman et al. 2002; Zhang et al. 2008). The bacterium *Achromobacter piechaudii*, isolated from dry riverbeds of southern

Israel, was capable of increasing salt and drought resistance in both pepper and tomato (Mayak et al. 2004). Using olive trees, tomato, grapevine, and pepper plants, Marasco et al. (2013) have demonstrated that microbes isolated from the roots of one host species cultivated under desert farming conditions are capable of improving the growth of a different host species when grown under a water-stress regime. The ability to transfer stress-resistance solutions from one crop species to another through a microbial inoculum has the potential to save years of plant breeding effort.

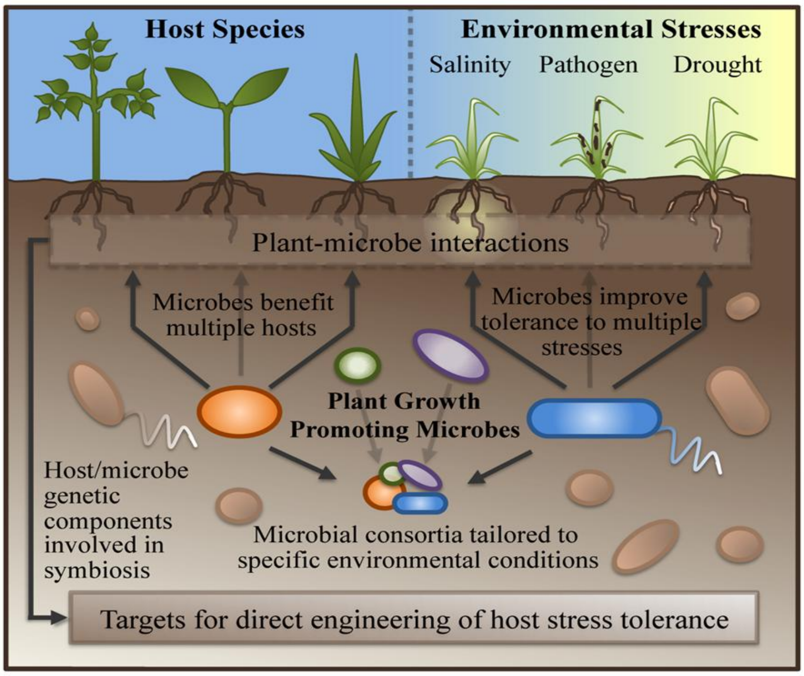

Fig. 2.2. Plant-growth promoting microbes are capable of conferring benefits to multiple species of plant hosts, and offering improved tolerance to multiple stresses simultaneously. Inoculations with combinations of PGPM can be tailored to specific environmental conditions. Dissection of plant–microbe interactions during symbiosis has the potential to reveal both the microbial and host genetic components responsible for improved stress tolerance; these may serve as targets for plant-breeding/genetic-engineering based approaches to improving stress tolerance in the host (Source: Coleman-Derr and Tringe 2014).

2.8. Rhizobacteria as salinity stress alleviators in crops with reference to Actinomycetes (actinobacteria)

In the last decade, bacteria belonging to different genera including *Rhizobium, Bacillus, Pseudomonas, Pantoea, Paenibacillus, Burkholderia, Achromobacter, Azospirillum, Microbacterium, Methylobacterium, Variovorax, Enterobacter* etc. have been reported to provide tolerance to host plants under different abiotic stress environments (Grover et al. 2011). Use of these microorganisms *per se* can alleviate stresses in agriculture thus opening a new and emerging application of microorganisms. Microbial elicited stress tolerance in plants may be due to a variety of mechanisms proposed from time to time based on studies done. Production of indole acetic acid, gibberellins and some unknown determinants by PGPR, results in increased root length, root surface area and number of root tips, leading to an enhanced uptake of nutrients thereby improving plant health under stress conditions (Egamberdieva and Kucharova 2009; Shrivastava and Kumar 2015). The mechanism of plant growth promotion by rhizobacteria has been shown in Fig 2.3.

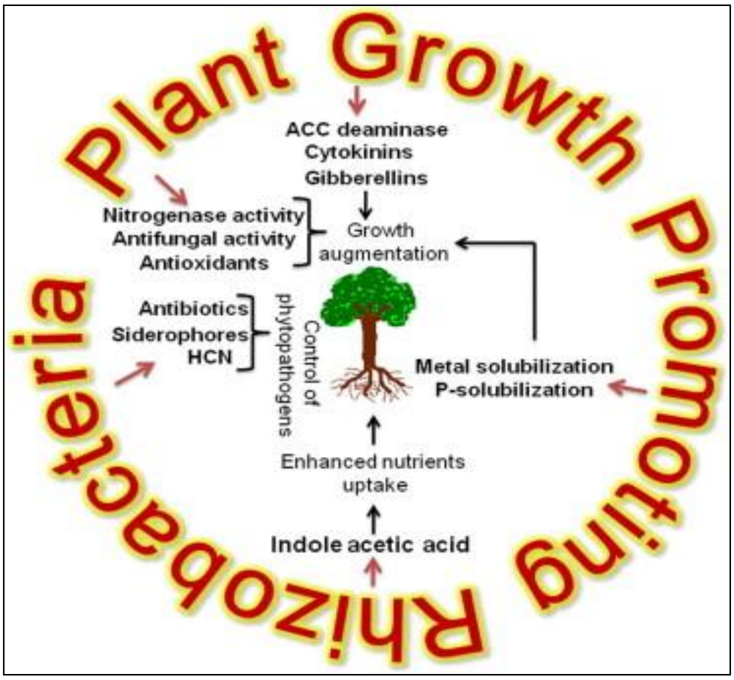

Fig. 2.3. Mechanism of plant growth promotion by rhizobacteria. (Source: Ahmad and Kibret 2014).

Plant growth promoting bacteria have been found to improve growth of tomato, pepper, canola, bean and lettuce under saline conditions (Barassi et al. 2006; Yildirim and Taylor 2005). Some PGPR strains produce cytokinin and antioxidants, which result in abscisic acid (ABA) accumulation and degradation of reactive oxygen species. High activities of antioxidant enzymes are linked with oxidative stress tolerance (Stajner et al. 1997). Another PGPR strain, *Achromobacter piechaudii* ARV8 which produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase, conferred induced systemic tolerance (IST) against drought and salt in pepper and tomato (Mayak et al. 2004). Upadhyay et al. (2011) studied the impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions and reported that co inoculation with *B. subtilis* and *Arthrobacter sp*. could alleviate the adverse effects of soil salinity on wheat growth with an increase in dry biomass, total soluble sugars and proline content. Jha et al. (2011) reported that *P. pseudoalcaligenes,* an endophytic bacterium in combination with a rhizospheric *B. pumilus* in paddy was able to protect the plant from abiotic stress by induction of osmoprotectant and antioxidant proteins than by the rhizospheric or endophytic bacteria alone at early stages of growth. Plants inoculated with endophytic bacterium *P. pseudoalcaligenes* showed a significantly higher concentration of glycine betaine-like quaternary compounds and higher shoot biomass at lower salinity levels. While at higher salinity levels, a mixture of both *P. pseudoalcaligenes* and *B. pumilus* showed better response against the adverse effects of salinity. Nia et al. (2012) studied the effect of inoculation of *Azospirillum* strains isolated from saline or non-saline soil on yield and yield components of wheat in salinity and they observed that inoculation with the two isolates increased salinity tolerance of wheat plants; the saline-adapted isolate significantly increased shoot dry weight and grain yield under severe water salinity. The component of grain yield most affected by inoculation was enhancement in grains per plant

Actinomycetes are one of the major microbial dominant groups and are well known for their saprophytic behaviour as well as for production of diverse bioactive secondary metabolites. They are also recognised for their capacity to survive in extreme habitats (Bredholdt et al. 2007). Members of alkali-halophilic actinomycetes are not much explored and are poorly understood. There are only a few reports available pertaining to actinomycetes from saline and alkaline habitats which can address the problem of hindered plant growth in salinity (Sadeghi et al. 2012). Recent findings from culturable and unculturable diversity have demonstrated that there are tremendous diversities and novelties among the halotolerant and alkaliphilic actinomycetes present in saline and alkaline environments (Chen et al. 2009; Luo et al. 2009; Malviya et al. 2014). In the past three decades, discoveries of new compounds have declined from soil derived actinomycetes (Fu et al. 2011). For this reason, researchers have started focusing towards unexplored niches like marine sources, lagoons, lakes and salt pans. They are exciting novel sources of bioactive compounds with promising anti-microbial activity against pathogenic strains (Cao et al. 2004; Castillo et al. 2007). Actinomycetes produce large number of antimicrobial compounds and plant growth promoting hormones are reported. There are many references cited with an ability of the *Streptomyces, Micromonospora, Corynebacterium, Frankia, Mycobacterium,* and *Rhodococcus* (Tsavkelova et al. 2005; Khamna et al. 2010) genera in plant growth promotion directly (production of phytohormones) (Hopwood and Chater 1990; Houssam 2009) or indirectly (production of cell wall degrading enzymes) (Walter and Crawford 1995). In spite of this, Streptomycetes is one of the dominant groups of microbes and plays a major role in environmental sustainability, while utilization of actinomycetes as salinity stress remediators is still in infancy. Sadeghi et al. (2012) studied the plant growth promoting activity of an auxin and siderophore producing isolate of *Streptomyces* under saline soil conditions and reported increases in growth and development of wheat plant. They observed significant increases in germination rate, percentage and uniformity, shoot length and dry weight compared to the control. Applying the bacterial inoculums increased the concentration of N, P, Fe and Mn in wheat shoots grown in normal and saline soil and thus concluded that Streptomyces isolate has potential to be utilized as biofertilizer in saline soils. The adverse effect of salinity in 'Micro tom' tomato was alleviated by *Streptomyces* strain PGPA 39 producing ACC deaminase, IAA and solubilising phosphate with salt tolerance capacity (Palaniyandi et al. 2014). In a recent study, performed by Krishnan et al. (2016) a novel halotolerant actinobacterial strain *Arthrobacter pokkalii* sp nov had been isolated with plant beneficial properties (*viz.* ACC deaminase activity, IAA and siderophore production) from saline tolerant pokkali rice rhizosphere, from Kerala, India.

As evident from the review, use of plant growth promotory actinobacteria for salinity stress alleviation is still in infancy; their diversity needs to be explored more especially in the saline affected IGP region which is a major region for wheat cultivation. This can help combat salinity induced reduction in wheat crop in these regions i.e. could have beneficial impact on the economy of the farmers in this region. With this background in mind, the present study was undertaken for isolation of halotolerant actinobacteria from the selected salinity affected districts (which may also add knowledge about the diversity of halotolerant actinobacteria in these districts), characterisation and their impact on wheat plant growth and yield.

Chapter 3

MATERIALS AND METHODS

Chapter 3

Materials and Methods

This chapter embodies the standard methods and protocols that were used to accomplish the objectives of the present study. All the ingredients and the media's used in the experiments were procured from Hi-media, India, SRL, India, Bangalore Genei, India, and Merck, India. The composition of various media is provided in Appendix, while some of the important media compositions are mentioned in the text. All the reagents used were of AR grade with 99.99% purity. Molecular studies were performed using standard protocols and the fine chemicals used were procured from Bangalore Genei, India and Promega, USA. Analytical instruments, their make and model are suitably mentioned in the text.

This chapter describes the soil sampling site used for isolation of the halophilic/halotolerant actinomycetes (microorganism used in the present study), their maintenance and characterization. A detail of pot culture experiment to study the interaction of halophilic actinomycetes in the growth enhancement of wheat under abiotic stress, i.e., salinity is also included in the present chapter. This chapter has been divided into sections and sub-sections wherever necessary for the ease of understanding.

3.1. Site description and soil sampling

The soil site selected for the isolation of halotolerant microorganisms used to isolate salinity stress in wheat was the Indo-Gangetic Plain (IGP) region of Uttar Pradesh, India, which is highly fertile with alluvial soil and pH ranging from 7.0 to 10.5. The topography of the region is flat with a slope of 2 m/km, and lies between 23°52' N to 31°28' N and 77°3' E to 84°39' E. The climate is predominantly sub-tropical, but variations exist because of differences in the altitudes. Depending on the elevation, the average temperatures vary from 12.5 to 17.5°C (55-64°F) in January to 27.5-32.5°C (82-91°F) in May and June. The temperatures rise to 45ºC, and sometimes may reach 47-48ºC during summers. There is low relative humidity of about 20% and the 85% of average annual rainfall of 990 mm. Salt-affected soils of Indo-

Gangetic alluvium plain (2.7 million ha) constitute centuries old barren alkali soils without any land-use system. A large tract of common lands (either government lands or village panchayat lands) are not in any productive use. Farming on the Indo-Gangetic Plain primarily consists of rice and wheat grown in rotation. Other crops include maize, sugarcane, pulses, oil seeds, potatoes, and cotton.

Salt-affected soil samples were collected from salinity compromised regions of IGP, *viz.*, Fatehpur, Kanpur, Auraiya, Etawah and Mainpuri districts of Uttar Pradesh state of India (coordinates 25^{o}93' to 27^{o}23'N and 79^{o}02' to 80^{o}13'E; Fig. 3.1). Sampling was done by random stratified method from 15-20 cm depth by removing the upper organic layer of soil using a sterile auger from each site in a sterile specimen container and the samples were immediately transported to the laboratory.

Fig. 3.1. Sites of collection of soil samples (Fatehpur, Kanpur, Auraiya, Etawah and Mainpuri districts) from Indo-Gangetic Plain regions of Uttar Pradesh.

For physico-chemical analysis, pulverized soil samples were mixed and homogenised. Then they were ground, air dried and passed through a 2.0 mm sieve. The samples were stored at 4°C and processed within 24 h of collection. The pH and EC of soil samples were measured in soil: water suspension (1:2.5 w/v) using a pH meter (Eutech Instruments, USA) and EC meter (Universal Bio, India), respectively, at room temperature (28°C) following the procedure outlined by Singh et al. (1999). Organic carbon was quantified using the Walkley and Black rapid titration method as modified by Walkley (1947). Other available nutrients in soil samples were analysed using soil testing kit (Himedia).

3.2. Isolation of actinomycetes (actinobacteria)

Soil samples were enriched with various techniques, *viz.*, $CaCO_3$ enrichment (1%; Tsao et al. 1960), SDS (5%) and phenol treatment (1.4%) (Hayakawa and Nonomura 1989; Hayakawa et al. 2004) so as to favour the growth of actinobacteria and inhibit fungi. Isolation of actinobacteria was done using standard serial dilution and plating techniques in triplicate on starch casein agar (SCA) and actinomycetes isolation agar (AIA) and humic acid vitamin agar (HVA), supplemented with actidione (50 mg mL^{-1}) so as to inhibit the growth of fungi (Goodfellow and Williams 1988) and 50 mg mL^{-1} nalidixic acid, which inhibits the bacteria capable of swarming without affecting the growth of actinobacteria (Nonomura and Hayakawa 1988). The air-dried samples were incubated at 55°C for 5 min. in an incubator. Stock solution was prepared by dissolving 1 g soil sample in 100 ml of distilled water (10^{-2} dilution) and mixed well using vortex mixer. From this initial suspension 1 ml was used to prepare the final dilutions of 10^{-3}, 10^{-4} and 10^{-5} by serial dilution technique. Finally, 0.1 ml of suspension from the dilutions 10^{-3}, 10^{-4} and 10^{-5} was spread plated on media amended with antibiotics using dilution-plate technique and incubated at 32±2°C for 2-3 weeks. The plates were observed periodically for the growth of actinomycetes. After incubation, actinomycetes colonies were selected and streaked on to the SCA media plates. The isolated strains were purified and maintained in slant culture (SCA slants) at 4°C, as well as 20% (v/v) glycerol stocks at -20°C.

3.3. Screening of the isolated actinomycetes for salt tolerance

The intrinsic tolerance of the actinobacterial strains against salinity was evaluated by observing the growth in starch casein agar amended with various concentrations of NaCl (2-10%) along with control plates maintained with 0.5% NaCl. The plates were incubated at 32±2^{o}C for 5-7 days (Yadav et al. 2009). Actinobacterial strains growing on more than 4% NaCl with optimum growth at 8% NaCl were selected for further study.

3.4. Morphological and physiological characterization of the halotolerant isolates

A. Morphological characterization

The selected halotolerant isolates were characterised morphologically based on cultural and morphological characteristics including colony colour and characteristics (cultural characterization), colour of aerial and substrate mycelium, spore mass colour, production of diffusible pigment and spore chain morphology on International Streptomyces project (ISP 2) media (Shirling and Gottlieb, 1966) according to Bergey's Manual of Determinative Bacteriology (Bergey and Holt 2000). Spore chain morphology and surface ornamentation was studied by Scanning electron microscopy (SEM). Mycelia were harvested (after 10 days of incubation) and washed in 0.1 M sodium cacodylate buffer (pH 7.4). They were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C followed by post-fixation with 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer (pH 7.4) and dried in a critical point dryer (Emitech K850, Hitachi, Japan). The preparations were mounted onto aluminium holders, sputter-coated with 10 nm Au and observed by SEM (Hitachi model S3400 at 15-30 kV, 2-5.00 μm) (Malviya et al. 2011).

B. Physiological characterization

Physiological characterization such as the effect of pH (5-11), temperature (25-55°C) and carbon source utilization were studied. Carbohydrate utilization was determined by growth on carbon utilization basal medium (Pridham and Gottlieb,

1948) supplemented with 1% carbon source (D-Glucose, D-Fructose, Xylose, Raffinose, Cellobiose, L-Arabinose, Mannose, D-Galactose, Inositol and Sucrose).

3.5. Biochemical characterization

Biochemical characterization of the halotolerant isolates was done by performing assays for extracellular enzyme production, hydrogen sulfide production and plant growth promotory attributes using standard protocols.

A. Extracellular enzymes and hydrogen sulfide production

Test organisms were studied for the production of various extracellular enzymes, which are summarized below:

i) Amylase production assay (Starch hydrolysis): Amylase activity was observed on Reese’s mineral media containing starch (1% w/v) as carbon source (Mishra and Behera 2008). Starch agar plates were prepared and the test isolates were spot inoculated on the plates following an incubation of 24-48 h at 37^{o}C. After incubation, the starch agar plate cultures were flooded with gram′s iodine solution for 30 seconds. The excess of iodine solution after staining was drained off. Development of blue black colour surrounding the growth of each test organism was examined. The blue colour of the medium indicates the absence of starch hydrolysis and a clear zone around the cultures indicates positive starch hydrolysis.

ii) Gelatinase production assay (gelatin degradation): For gelatin degradation, the nutrient gelatin tubes were prepared and using sterile technique, each test isolate was inoculated into the tubes by means of a stab inoculation (Cappuccino and Sherman 2005). All the tubes were incubated for 24 to 48 h at 37^{o}C. After completion of incubation, gelatin tube cultures were placed into a refrigerator at 4^{o}C for 30 min. All the tubes were then examined to determine whether the medium is solid or liquid. Liquefaction of the medium indicates of positive gelatinase assay while solid medium indicates a negative gelatinase assay.

iii) Urease production assay (urea hydrolysis): The urease activity of the isolates was tested on basal medium amended with 1% urea (Cappuccino and Sherman

2005). Solution of 1% urea was prepared by dissolving 10 g urea in 100 ml sterile water and filtered through bacterial filters and stored in sterile vials. Filter sterilized urea was then added to the autoclaved luke warm basal medium aseptically and poured into sterile test tubes. The cultures to be tested were inoculated in the test tubes and were incubated for 24 to 48 h at 37^oC. Urea hydrolysis was determined by pink coloration of the medium. Change of media colour from yellow to pink shows a positive urease activity.

iv) Protease production assay: The protease activity was determined using skim milk agar medium (composition in gL^{-1}: 5, pancreatic digest of casein (Hi Media, India); 2.5, yeast extract; 1, glucose; 25, skim milk; and 15, agar) (Manachini et al. 2008). The actinomycetes isolates were streaked on the skim milk agar media plates and proteolytic activity was identified by a clear zone around the cells after an incubation of three days at 37°C. Formation of clear halo around the colonies indicates a positive protease assay.

v) Cellulase production assay (cellulose hydrolysis): Cellulolytic activity was observed by Reese’s mineral media containing cellulose (1% w/v) as carbon source (Routella and Cowling, 1966; Farkas et al. 1985). The isolated halotolerant cultures were streaked on alkaline carboxyl methyl cellulose (CMC) agar plates and were incubated at 37^oC for 3-4 days. The plates after incubation were flooded with 0.1% Congo red dye solution and were kept for 15 min. After 15 min, the Congo red dye was drained off and the plates were washed twice with 10mM NaCl solution to observe halo zones. The presence of halo zones represents the hydrolysis of cellulose.

B. Hydrogen Sulfide (H_2S) Production (Sulphate reduction): SIM (Sulfide indole motility) agar media was prepared for examining the H_2S production (Cappuccino and Sherman 2005). The 10 ml of media was dispensed in to test tubes and autoclaved. The Actinomycetes cultures were stab inoculated in the tubes and incubated for 24 to 48 h at 37^oC. Stab cultures of all actinomycetes isolates on SIM agar tubes were observed for the presence or absence of black colouration along the line of stab inoculation. Black coloration along the stab indicates the production of hydrogen sulfide.

C. Plant growth promotory (PGP) traits

The halotolerant isolates were screened for direct [Indole acetic acid (IAA) production and phosphate solubilization] and indirect (Production of ammonia, siderophore, HCN, chitinase and biocontrol against *Macrophomina phaseolina* and *Sclerotium rolfsii*) plant growth promotion traits.

Estimation of direct PGP attributes

i) Indole Aceitc Acid (IAA) production: One hundred microliter spore suspension (10^6 cfu/ml) was inoculated in 50 ml conical flasks containing 10 ml of glucose yeast extract malt extract broth supplemented with graded series of NaCl concentrations (0, 2, 4, 6, 8% w/v) in triplicates and were allowed to grow in shaking incubator for five days at 32^oC. Cells were then collected by centrifugation at 12,000 rpm for 15 min. Finally, 2 ml of Salkowsky's reagent was added to the supernatant and further incubated for 25 min in dark at 30°C (Patten and Glick 2002). IAA production was observed as the development of a pink-red colour. The absorbance of the pink coloured complex was read at 530 nm in a UV–Vis Spectrophotometer (Schimadzu, Japan). The calibration plot was constructed using dilutions of a standard IAA (HiMedia, India) solution and the uninoculated medium with the reagent as a control. The quantity of IAA produced in the broth culture was expressed as µg mg^{-1} protein.

Estimation of protein: The cells left after taking supernatant were resuspended in 1N NaOH (5 mL), boiled for 30 min, cooled, and centrifuged at 10,000 rpm for 5 min. The protein concentration was determined by the method of Lowry et al. (1951). 0.1 mL of the protein sample was extracted out into separate test tube, and the final volume was made to 1 mL with distilled water. A tube filled with 1 mL of distilled water served as the blank. 5 mL of the reagent C (Appendix III) was added to each tube including the blank, and mixed well. The mixture was allowed to stand for 10 min. Then, 0.5 mL of reagent D (Folin-Ciocalteau) was added, followed by mixing, and then incubated at room temperature in the dark for 30 min. The absorbance of the blue coloured complex developed was taken at 660 nm.

ii) Phosphate solubilisation: Qualitative phosphate solubilization activity of potential halotolerant actinomycetes isolates were analyzed on Pikovskaya (PKV) agar media (Pikovskaya 1948). The cultures were spot inoculated on PKV agar media plates and incubated at 37^{o}C. Those forming clear halo zone around their colonies after five days incubation were considered to have phosphate solubilization capability.

Estimation of indirect PGP attributes

i) Production of ammonia: For determination of ammonia production, 20 μl of seed culture was propagated in 10 ml of peptone water broth in test tubes and incubated at 37°C with shaking at 120 rpm for five days (Cappuccino and Sherman 2005). Subsequently, 0.5 ml of Nesseler's reagent was added to the test tubes, development of brown to yellow colour indicates a positive test for ammonia production.

ii) Siderophore production: To detect the production of siderophores, method of Schwyn and Neiland (1987) was used. The isolates were spot inoculated on to Chrome-azurol S (CAS) agar media plates and incubated at 32±2^{o}C for 2-6 days. The plates were observed for orange halo formation around actinomycetes colonies after six days. This orange halo formation indicates positive siderophore production.

iii) HCN production: The isolates were assayed for HCN production by the method of Bakker and Schippers (1987). Nutrient broth amended with 4.4 gm/l of glycine was distributed in test tubes and inoculated with test culture. One sheet of Whatman filter paper no. 1 (8 cm diameter) was soaked in 1% picric acid (in 10% sodium carbonate). The filter paper and picric acid were sterilized separately for a minute, thereafter; the filter paper was soaked in picric acid. The soaked filter paper strip was placed in each test tube by hanging it from the cotton plug. The test tubes were sealed with parafilm and incubated at 32^{o}C for four days. Development of a reddish brown colour on the filter paper strip indicates HCN production.

iv) Chitinase enzyme activity: Chitinase enzyme activity of the selected isolates was tested on the colloidal chitin agar medium (Skujins et al. 1965). Actinomycetes cells were spot inoculated and chitinase activity was identified by clear zone around the cells after incubation at 32±2°C for five days. A clear halo zone on colloidal chitin agar medium shows positive chitinase activity.

v) Biocontrol against fungal pathogen: This test was performed using dual culture assay and direct interaction of fungi and the test actinobacteria using SEM. Both the methodologies are described below:

Dual culture assay: Each of the 28 halotolerant isolates were evaluated for their *in vitro* antifungal activity by dual culture assays on potato dextrose agar (PDA; Hi-Media, India) against the two selected plant pathogens namely, *Sclerotium rolfsii* (SR) and *Macrophomina phaseolina* (MP) (Bredholdt et al. 2007). A fungal disc (SR/MP) of 5 mm diameter taken from an actively growing culture was placed at centre on the surface of the PDA plate. Actinomycetes strains grown in ISP-2 broth to a concentration of (~$6x10^8$ cell mL^{-1}) were streaked in a straight line on the other edge of the PDA plate (3 cm from the centre) containing fungal disk, followed by incubation at 28±2^{o}C for 5 days or till the fungal mycelia completely covered the entire plate in the control. The plate inoculated with fungal disk alone was used as control. The percent inhibition was calculated using the formula C-T/C × 100, where, C is the colony growth of fungal pathogen in control, and T is the colony growth in dual culture. All experiments were carried out in triplicate.

Interaction study of biocontrol agent with test pathogen through Scanning electron microscopy (SEM): The interaction of the plant pathogenic fungi (*SR/MP*) with the most potent antagonistic isolates was studied by scanning electron microscopy (SEM). The mycelial disc (8 mm) from the area of interaction zone was cut and transferred on glass cover slips, fixed with 2% glutaraldehyde and subjected to (60, 70, 80 and 90%) serial alcoholic dehydration treatment (Islam et al. 2005). The specimens were dried in a critical point dryer (EMITECH K850, Hitachi) and affixed to SEM stubs using carbon tape followed by thin coating with gold: palladium (60:40) and observed under SEM (Hitachi, S3400 at 15-30 kV, 2-5.0 µm).

3.6. Molecular characterization and identification of halotolerant isolates

i) Genomic DNA extraction

The actinomycetes cultures were grown in 150 ml ISP-2 broth and gyrated at 125 rpm for 3-5 days at 32±2^{o}C. The cells were harvested by taking 1ml of culture broth into 1.5 ml tube and discarding the supernatant after centrifugation at 12,000 rpm for 10 min. Total genomic DNA was extracted, as described by Boudjella et al. (2006), with slight modifications (Appendix IIB).

The isolated DNA was observed and quantified by agarose gel electrophoresis. 5 µL of DNA preparation of all actinomycetes isolates along with the standard DNA ladder (Genei) were electrophoresed on 0.8% agarose gel prepared in 1X TBE Buffer. Electrophoresis was carried out at 50 volts for 90 minutes (Sambrook and Russel 2001). The DNA was stained by ethidium bromide, visualized on UV transilluminator and documented by Bio-Rad digital gel documentation system. The quality was checked from the appearance of the bands. Appearance of sharp bands was considered as good quality DNA preparation. The quantity was determined from the known concentrations of DNA marker as a standard. After quantification, the DNA was diluted by molecular grade water (Protease, Nuclease free; Bangalore GeNei, Bangalore, India) or TE buffer to the final concentration of 50 ng/µL.

ii) Amplification of the 16S rRNA gene

PCR amplification of 16S rRNA gene was performed from the genomic DNA of the actinomycete strains using actinomycetes specific universal primers (McVeigh et al. 1996): forward primer ACT283F (5'-gggtagccggccugagaggg-3') and reverse primer 1360R (5'-ctgatctgcgattactagcgactcc-3'). PCR cocktail (100 µL) contained 2.5 µL of each primer (10 pM), 4-6 µL of genomic DNA, 10 µL of 10× *Taq* DNA polymerase buffer, 1U of *Taq* DNA polymerase (3U/µL) (Banglore Genei, India), 2 µL of 2.5 mM of each dNTP, and the final volume was made up with nuclease free water. Amplification was performed in a DNA thermo cycler (Bio-Rad Laboratories, CA, USA) at 95°C for 5 min, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C and

1 min. at 72°C with an final extension of 72°C for 5 min. A 5µl aliquot of each amplified product was electrophoresed on a 1.2% agarose gel along with 1 kb DNA ladder as marker in 1× TAE buffer at 50V for 45 min, stained with ethidium bromide and the PCR products were visualized with a UV transilluminator (Bio-Rad Laboratories, CA, USA).

iii) Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA), was employed as a tool to study microbial diversity that relied on DNA polymorphism. The amplicons generated by PCR were digested separately with three restriction endonucleases: *Msp*I, *Hae*III and *Taq*I (Table 3.1). Restriction was carried out in a 25 µL reaction mixture containing 1.2 µL of restriction enzyme (5U µL^{-1}), 2.5 µL of dilution buffer (10×), 12 µL of PCR product and Milli-Q water (protease, nuclease free) added to make up the volume and incubated at 37^{o}C, except for *Taq*I at 65 ^{o}C for 4 h. The digested fragments were separated by electrophoresis on 2.5% agarose gel containing ethidium bromide (10 mg mL^{-1}) and photographed under ultraviolet light (Sambrook and Russel 2001).

Table 3.1. Restriction enzymes used for RFLP analysis

S. No	Restriction enzyme	Recognition site
1	*Msp* I	**C$^{\downarrow}$CGG**
2	*Hae* III	**GG$^{\downarrow}$CC**
3	*Taq* I	**T$^{\downarrow}$CGA**

Fragment sizes were compared with 100 bp DNA ladder (GeNei) used as molecular size marker. The analysis was done at least twice with each enzyme. Strong and clear bands were scored for similarity and clustering analysis using the software, NTSYS-2.02e package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the strains was calculated by Jaccard's coefficient (Jaccard, 1912) and dendrogram was constructed using UPGMA method (Nei and Li, 1979).

iv) Partial sequencing and phylogenetic analysis

PCR products were purified using PCR purification kit (Promega, India) and sequenced directly with the *Taq*- mediated Di-deoxy chain terminator cycle sequencing kit (Applied Biosystem, India) in an ABI 3130*xl* automated genetic analyser (Applied Biosystem, UK) according to manufacturer’s instructions (Applied Biosystems). The primers used for sequencing were the same set of primers as used in PCR amplification. 16S rDNA amplicons were sequenced from both ends and consensus sequence was generated.

To perform molecular phylogenetic analysis, reference sequences were downloaded from the NCBI database (GenBank) by BLASTn search and identification to the species level was determined on the basis of sequence similarity of ≥98% with the closest relative in the GenBank. All the sequences of 16S rRNA gene were aligned using the multiple sequence alignment program CLUSTALW (Larkin et al., 2007). The aligned sequences were then checked for gaps and saved as molecular evolutionary genetics analysis (MEGA) format in software MEGA v4.1. The pair wise evolutionary distances were calculated using the Kimura 2-parameter model (Kimura, 1980). To obtain the confidence values, the original data set was resampled 1000 times using the bootstrap analysis method. The bootstrapped data set was used directly for constructing the phylogenetic tree using the MEGA v4.1 program for calculating the multiple distance matrixes (Tamura et al., 2007). The multiple distance matrix obtained was then used to construct phylogenetic trees using neighbour-joining (NJ) method (Saitou and Nei, 1987).

v) Nucleotide Sequence Accession Numbers

The nucleotide sequences of actinomycetes specific 16S rRNA gene were deposited in NCBI GenBank. The accession numbers and amplicon size (bp) of 16S rRNA gene nucleotide sequences of the strains are presented in results.

3.7. PCR amplification for hydrolytic enzyme gene chitinase (*chiA*)

PCR amplification of chitinase *(chiA)* gene was performed using the PCR primers: F-5′-gatatcgactgggagttccc-3′ and R-5′-catagaagtcgtaggtcatc-3′ for *chiA* gene

(Ramaiah et al. 2000). The amplification was performed in a 100 µL reaction volume, following reaction mixtures and conditions as specified in Table 3.2. The amplified PCR products were resolved on 1.2% agarose gel with 100 bp ladder.

Table 3.2. PCR requirements for amplification of hydrolytic enzyme gene chitinase (*ChiA*)

A. Reaction mixture	**Quantity**
10× Taq buffer	10 µL
$MgCl_2$ (1.5mM)	1.5 µL
BSA (20 mg mL^{-1})	0.2 µL
DMSO (100%)	-
Taq polymerase (3 U)	1 µL
dNTP mixture (10 mM)	6 µL
Primer (Forward)	14.6 µL
Primer (Reverse)	14.6 µL
Template DNA	4 µL
Water (Molecular grade)	48.1 µL
B. Reaction conditions	**Temperature and duration**
Heated lid	110°C
Initial temperature	94°C for 5 min.
Start cycles	-
Number of cycles	30
Denaturation	92°C for 30 sec.
Primer annealing	58°C for 30 sec.
Primer elongation	72°C for 90 sec.
End cycles	-
Final extension	72°C for 7 min.

3.8. Estimation of osmolyte (proline) production

Production of proline, which is an osmolyte, and helps the organism to tolerate high salinity, was tested using the method of Bates et al. (1973). Proline content was estimated based on proline's reaction with ninhydrin. Briefly, 5 days old cultures grown in broth amended with different concentrations of NaCl (0, 2, 4, 6% w/v NaCl) were centrifuged at 12,000 rpm for 5 min and the supernatant was homogenized with 3% sulfo-salicylic acid. For proline colorimetric determinations, a 1:1:1 solution of supernatant, ninhydrin acid and glacial acetic acid was incubated at 100 °C for 1 hour. The reaction was arrested in an ice bath and the chromophore was extracted with 4 ml toluene and its absorbance at 520 nm was measured in spectrophotometer (Schimadzu, Japan) and the calibration curve was determined using pure L-proline (Himedia, India) as standard.

3.9. *In vivo* plant growth promotion assay under non-saline and saline conditions with wheat (*Triticum aestivum* L.)

i) Selection of actinomycetes strain for *in vivo* assay: The two most potent halotolerant actinomycetes isolates *Streptomyces albogriseolus* (K34) and *S. griseorubens* (K36) were selected based on their IAA production and siderophore production capabilities under stress conditions and the potential to produce compatible solute- proline for stress remediation. Since the isolates were siderophore producers, so the quantity of siderophore produced was also estimated spectrophotometrically under normal and stressed condition as follows: Quantification of siderophore was done following protocol of Schywn and Neilands (1987); Payne (1994) with some modifications. Cultures were grown in Glucose Yeast Extract Malt Extract broth at 32 °C for 72 hours under static conditions in new glasswares in iron free condition, supplemented with different concentrations of NaCl (0, 2, 4, 6% w/v). The cells were removed by centrifugation at 8,000 rpm for 15 min. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS (chrome azurol S) solution and 10 µl shuttling solution (sulfo-salicylic acid). The colour obtained was determined using the spectrophotometer at 630 nm after 20 mins of incubation. Necessary blank (Glucose Yeast Extract Malt Extract broth) and reference solution (Glucose Yeast Extract Malt Extract broth + CAS dye + shuttle solution) were used during the determination. The values of the siderophore produced were determined using the formula $[(Ar - As/Ar) \times 100]$ which gives percent siderophore units (Ar – absorbance of reference; As – absorbance of sample).

ii) Inoculum Preparation: The isolates were grown on ISP-2 broth at 32°C for 5 d with continuous shaking at 150 rpm. The cells were centrifuged at 10,000 rpm for 15 min and the pellets obtained, were diluted with distilled water to yield a final concentration of 10^8 CFU/ml. The halotolerant actinomycetes suspension was used to treat targeted plants under natural environmental conditions in a pot assay by mixing with sterilized soil.

iii) Wheat seeds and their sterilization: Wheat seeds (*Triticum aestivum* L. var. HUW 468 /Malviya 468) were procured from Institute of Agricultural Sciences, Banaras Hindu University (BHU), Varanasi, Uttar Pradesh, India. The seeds were surface sterilized by a very short immersion (30-60s) in 95% ethanol solution followed by immersion in a 0.2% $HgCl_2$ solution for 2 min, and then washed thoroughly with sterilized distilled water to remove all the traces of remaining $HgCl_2$.

iv) *In vivo* assay in non-saline condition: The sterilized wheat seeds were sown (5 seeds per cup) in small plastic cups (wiped with 95% ethanol) containing sterilized sand. Four treatments *viz.* control (no isolate added only sand), T1 (Sand + isolate K34), T2 (Sand + isolate K36), T3 (Sand + isolate K34 + isolate K36) were maintained in triplicates. Prior to sowing seeds, the actinomycetes inocula was mixed with the filling and after sowing, the cups were kept in natural environmental condition in completely randomized manner. The cups were watered on alternate days with sterilized distilled water. After 15 days of sowing, the shoot length and root length of wheat seedlings and proline synthesis was noted down.

v) *In vivo* assay under saline condition: The experiment was conducted in plastic pots (30 cm diameter) containing 9 kg of autoclaved (121^oC, 60 min; 1.5 air pressure for 3 consecutive days) saline-sodic soil collected from Babasaheb Bhimrao Ambedkar University campus with following properties: pH 9.2, EC 4.62 dSm^{-1}, organic C 0.21%. Soil was passed through a 2 mm sieve before filling into the pots. Pots were sterilized by wiping using 95% ethanol solution. Four treatments were maintained (control, T1, T2 and T3) similar to non-saline condition. Initially, 15 seeds were sown in each pot, and after germination 10 uniform seedlings were maintained and the pots were irrigated with sterilized distilled water. All the treatments were replicated thrice in completely randomized manner and the pots were kept in natural environmental conditions. Three plants were uprooted carefully from pots 15 and 30 days after sowing, to record shoot length, root length and proline accumulation in seedlings. At final harvest, vegetative and yield parameters (no. of tillers/plant, no. of spikes/plant, spike length, no. of spikelets/spike, grain yield and straw yield) of wheat were also recorded.

3.10. Statistical analysis

The data was subjected to analysis of variance (ANOVA) and subsequently by Duncan's multiple range tests by using the SPSS 16.0 software (SPSS Inc., Chicago, IL). The differences in the mean values in each treatment were compared and significance level was calculated at $P \leq 0.05$ levels for each treatment. Phylogenetic tree of 16S rDNA sequence was constructed by neighbour joining method and bootstrap analysis was carried out using MEGA version v4.1. Identification to the species level was determined as maximum homology (≥97%) to a prototype strain sequence in the GenBank. Phylogenetic analyses of PCR-RFLP (ARDRA) fingerprints were performed by positions of unequivocally scorable bands. The bands were transformed into a binary character matrix ("1" for the presence and "0" for the absence of a band at a particular position). Likewise, data in the form of binary code was prepared as worksheet in MS-excel and pairwise distance matrices were compiled by the NTSYS 2.02e software using the Jaccard's coefficient of similarity. Phylogenetic tree was created by the un-weighted pair-group method arithmetic (UPGMA) average cluster analysis (Saitou and Nei, 1987).

Chapter 4

RESULTS

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Results

4.1. Soil characteristics

A total of twenty soil samples (four samples each from five different collection sites) were randomly collected from salinity affected soils of Indo-Gangetic Plains (IGP) of India, *viz*., Fatehpur, Kanpur, Auraiya, Etawah and Mainpuri districts of Uttar Pradesh state. The textures of the soil samples were sandy, loam and clay. The pH of the soil samples ranged from 7.4 to 10.8 indicating their alkaline nature. Similarly, electrical conductivity (EC) of soil was recorded between 2.24 and 13.26 dSm^{-1}, which represents slightly saline to highly saline-sodic status. Among all, soil samples from Fatehpur showed the maximum (5.24-13.26 dSm^{-1}) EC value, while the minimum was recorded for soil samples of Auraiya (2.56-4.12 dSm^{-1}). The EC of the soil samples is highly dependent on soil particle size and texture.

The organic carbon content of the different soil samples ranged from 0.48 to 0.69; the lower carbon content (0.48-0.54) was recorded for soils from Fatehpur region, while the soil samples from Etawah (0.52-0.61) and Mainpuri (0.54-0.64) though possessed better organic carbon percentage but was less than OC % possessed by soils of Kanpur (0.58-0.69) and Auraiya (0.61-0.68). The available soil nitrogen, phosphorus and potassium were measured in $kgha^{-1}$. The soil samples of all the five sampling locations recorded very low (<22 $kgha^{-1}$) phosphorus level. The available amount of nitrogen in the soil was least in the soil samples of Mainpuri (~4 $kgha^{-1}$) and very low to medium in soil samples of Fatehpur (4-20 $kgha^{-1}$). While in other sites, *viz*., Kanpur it was low and in Auraiya and Etawah, the available amount of nitrogen was very low to low. The range of available potassium was medium (112-280 $kgha^{-1}$) for soils from Kanpur and Etawah to high (280-392 $kgha^{-1}$) for Fatehpur, Auraiya, and Mainpuri districts. The details of sampling sites, soil properties and other important soil characteristics are provided in Table 4.1.

Table 4.1. Details of sampling sites, soil properties and other important soil characteristics.

Parameter	Sampling sites (District of Uttar Pradesh, India)				
	Fatehpur	**Kanpur**	**Auraiya**	**Etawah**	**Mainpuri**
Geographical location	80°8'E, 25°93'N	80°13'E, 26°30'N	79°52'E, 26°47'N	79°03'E, 26°77'N	79°02'E, 27°23'N
No. of soil samples	04	04	04	04	04
pH range	8.6-10.8	7.8-9.9	8.0-8.5	7.4-8.8	7.5-9.0
EC (dSm^{-1})	5.24-13.26	3.13-4.42	2.56-4.12	2.24-5.81	4.17-5.48
Organic carbon (%)	0.48-0.54	0.58-0.69	0.61-0.68	0.52-0.61	0.54-0.64
Available P (kg/ha)	Very less (<22)	Very less (<22)	Very less (<22)	Very less (<22)	Very less (<22)
Available N (kg/ha)	Very low-medium (4-20)	Low (~10)	Very low-low (4-10)	Very low-low (4-10)	Very low (~4)
Available K (kg/ha)	High (280-392)	Medium (112-280)	High (280-392)	Medium (112-280)	High (280-392)

4.2. Isolation of actinomycetes and screening for salt tolerance

A total of fifty five actinomycetes isolates were isolated from composite saline soil samples using the various enrichment techniques and isolation media’s, viz., Actinomycetes isolation agar (AIA), starch casein agar (SCA), and Humic acid vitamin agar (HVA) media. Although, each of the three isolation media supported the growth of actinomycetes, the starch casein agar media supported better growth of actinomycetes colonies (Fig. 4.1). Amendment of antifungal agent actidione (50 mg mL^{-1}) inhibited the growth of fungal colonies. All the 55 actinomycetes isolates were screened for salt tolerance on SCA media amended with 0 to 10% of sodium chloride (NaCl) and the results showed that out of the 55 isolates, 28 isolates could tolerate a salinity level upto 8% (w/v) NaCl, while eighteen of the isolates could tolerate and grow upto 10% NaCl (Table 4.2, Fig. 4.2).

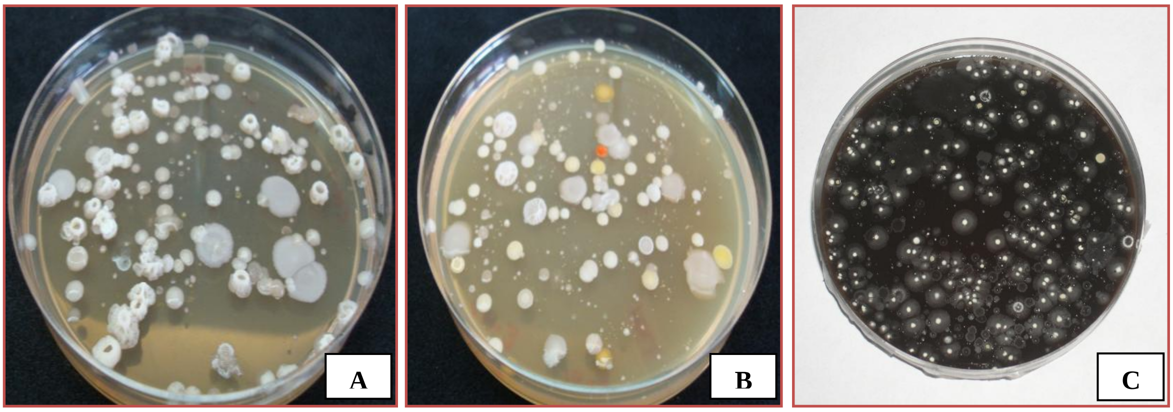


Fig. 4.1. Representative isolation plates containing different isolation media; Actinomycetes isolation agar (AIA) media: A; Starch casein agar (SCA) media: B; and Humic acid vitamin agar (HVA) media: C.

Table 4.2. Salt tolerance assay and number of halotolerant isolates that were obtained at different NaCl concentrations.

Salinity level (NaCl; %)	Number of halotolerant isolates
0.0	55
2.0	44
4.0	42
6.0	34
8.0	28
10.0	18

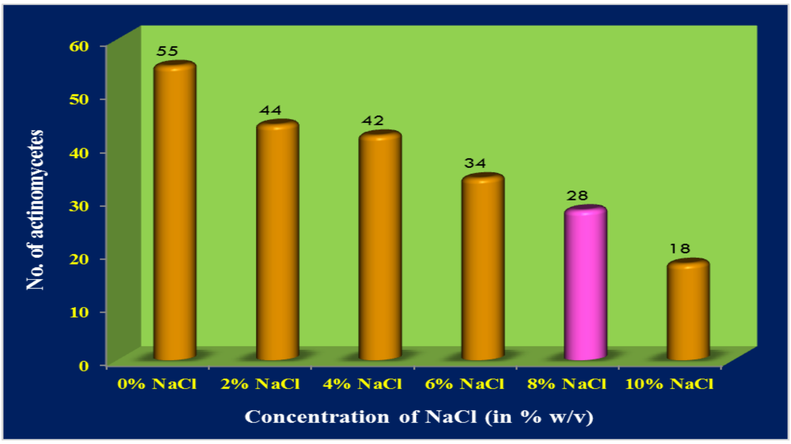


Fig. 4.2. Number of halotolerant isolates at different NaCl levels (pink bar indicates halotolerant isolates that were selected for further studies).

The twenty eight halotolerant isolates which showed good growth in 6-8% of NaCl salt concentrations were selected for further characterisation studies. Since these twenty eight isolates exhibited good growth in absence or presence of salt concentrations, such isolates were designated as moderately (intermediate) halotolerant.

4.3. Characterization of selected halotolerant isolates

i) Morphological and physiological characterisation

After screening, a total of 28 halotolerant isolates were selected and characterized morphologically on International Streptomyces Project 2 (ISP 2) media. All the 28 isolates exhibited good growth with hard/embedded colonies on media. The colours of aerial and substrate mycelia were found to vary from isolate to isolate. Of the twenty eight isolates, seventeen (K8, K9, K10, K13, K14, K17, K20, K21, K24, K26, K29, K30, K31, K32, K34, K37, K38) were reported to have aerial mycelia bearing grey spore mass. Whereas, four isolates (K2, K3, K5, K33) had aerial mycelia of pink spores, three isolates (K4, K16, K35) bore creamy spore mass, two isolates (K12 and K22) white aerial spore mass, one isolate (K28) showed ivory aerial spores and one (K36) had ash brown aerial spore mass. The substrate mycelium colour in nine (K2, K4, K8, K12, K16, K22, K29, K32 K33) isolates was mostly yellow or a variant of yellow. In contrast, seven isolates (K9, K13, K14, K21, K31, K35, K36, K38) had brown or beige-brown colour. Three isolates (K10, K20 and K24) had off-white/pale white substrate mycelia; yellow brown coloured substrate mycelium was reported in three isolates (K26, K28 and K34). Isolates K17 and K37) had beige, while isolates K3 and K30 had colourless substrate mycelia. One of isolates, *viz.*, K35 showed off white substrate mycelia.

Soluble pigment production was reported in five isolates (K21, K32, K35, K36 and K38). The detailed morphology of isolates is represented in Table 4.3 and Fig. 4.3. Scanning electron microscope (SEM) studies for spore ornamentation revealed that most of the isolates had smooth spores (Table 4.3). However four isolates, *viz.*, K4, K5, K9 and K24 had spiny spores while one isolate (K30) had hairy spore morphology. The spore chain varied in different isolates including: straight, rectiflexibilis, retinaculiperti and spirals spore chain (Fig. 4.4).

Table 4.3. Morphological characteristics of actinomycetes strains on glucose yeast extract malt extract agar (ISP 2) media at 30°C incubation after 7 days.

Isolate code	**Growth pattern**	**Mycelium colour**		**Soluble pigment**	**Colony**		**Spore surface ornamentation**
		Substrate	**Aerial**		**Consistency**	**Shape**	
K2	Good growth	Lemon yellow	Pink	None	Hard/embedded	Circular	Smooth spores
K3	Good growth	Colourless	Pink	None	Hard/embedded	Circular	Retinaculiperti smooth spores
K4	Good growth	Yellow orange	Cream	None	Hard/embedded	Circular	Spiral spiny spores
K5	Good growth	Creamish	Pink	None	Hard/embedded	Circular	Spiny spores
K8	Good growth	Sand yellow	Grey	None	Hard/embedded	Circular	Spirals smooth spores
K9	Good growth	Brown	Grey	None	Hard/embedded	Circular	Spirals with spiny spores
K10	Good growth	Off white	Grey	None	Hard/embedded	Circular	Retinaculiperti smooth spores
K12	Good growth	Yellow	White	None	Hard/embedded	Circular	Smooth spores
K13	Good growth	Light brown	Grey	None	Hard/embedded	Circular	Spirals smooth spores
K14	Good growth	Brown-beige	Light grey	None	Hard/embedded	Circular	Spirals with smooth spores
K16	Good growth	Maize yellow	Cream	None	Hard/embedded	Circular	Smooth spores in spirals
K17	Good growth	Beige	Grey	None	Hard/embedded	Circular	Rectiflexibilis smooth spores
K20	Good growth	Pale white	Grey	None	Hard/embedded	Circular	Rectiflexibilis smooth spores
K21	Good growth	Brown	Beige-grey	Present	Hard/embedded	Circular	Smooth spirals
K22	Good growth	Light yellow	White	None	Hard/embedded	Circular	Smooth spores
K24	Good growth	Off white	Dark grey	None	Hard/embedded	Circular	Spiny spores
K26	Good growth	Yellow brown	Grey	None	Hard/embedded	Circular	Rectiflexibilis smooth spores
K28	Good growth	Yellow-brown	Ivory	None	Hard/embedded	Circular	Rectiflexibilis smooth spores
K29	Good growth	Yellow	Grey	None	Hard/embedded	Circular	Smooth spores

K30	Good growth	Colourless	Grey	None	Hard/embedded	Circular	Retinaculiperti hairy spores
K31	Good growth	Light brown	Grey	None	Hard/embedded	Circular	Spiral smooth spores
K32	Good growth	Sand yellow	Grey	Present	Hard/embedded	Circular	Spiral smooth spores
K33	Good growth	Lemon yellow	Pink	None	Hard/embedded	Circular	Retinaculiperti smooth spores
K34	Good growth	Yellow brown	Grey	None	Hard/embedded	Circular	Rectiflexibilis smooth spores
K35	Good growth	Beige-brown	Cream	Present	Hard/embedded	Circular	Smooth spores in spirals
K36	Good growth	Brown	Ash brown	Present	Hard/embedded	Circular	Spiral smooth spores
K37	Good growth	Beige	White turning to grey	None	Hard/embedded	Circular	Rectiflexibilis smooth spores
K38	Good growth	Brown	Dark grey	Present	Hard/embedded	Circular	Straight, smooth spores

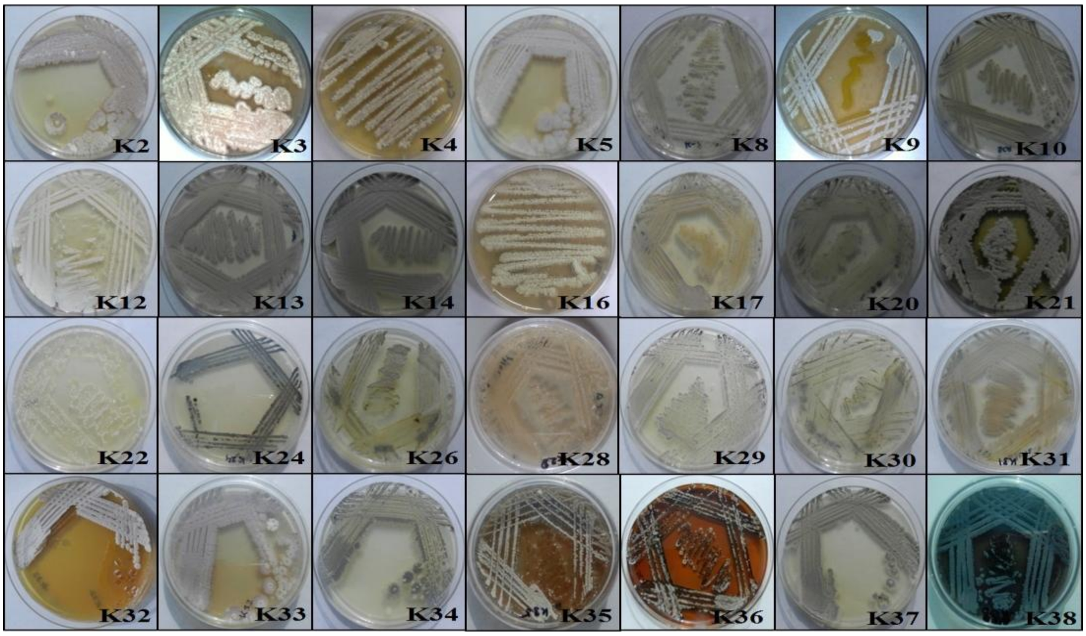
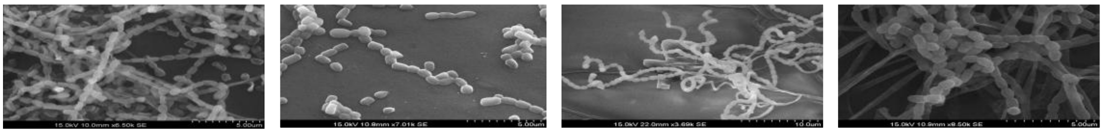
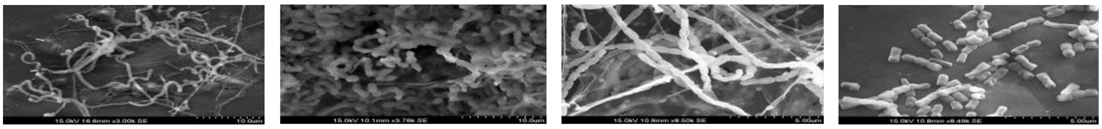


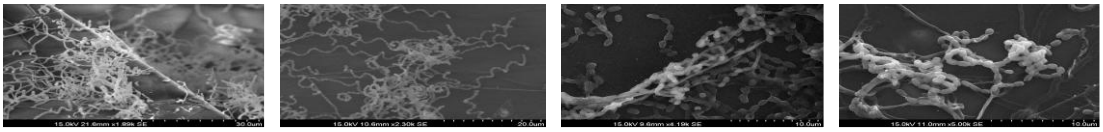
Fig. 4.3. Colony morphology of 28 halotolerant actinobacterial isolates isolated from IGP, India.



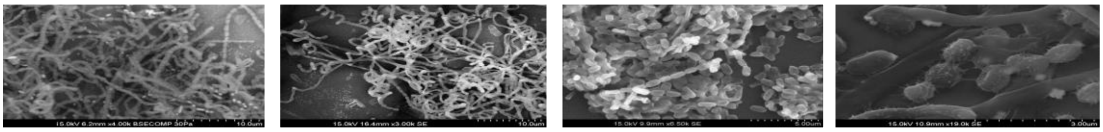
(K2) *S. fradiae*
(K3) *S. fradiae*
(K4) *S. coeruleorubidus*
(K5) *S. thermolilacinus*



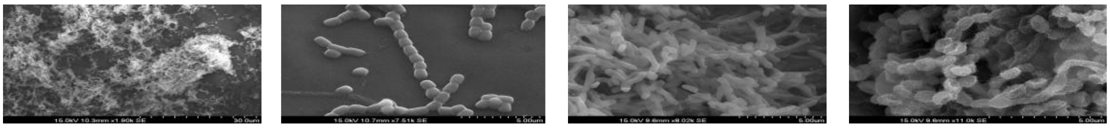
(K8) *S. atrovirens*
(K9) *S. gancidicus*
(K10) *S. thermoluteus*
(K12) *S. celluloflavus*



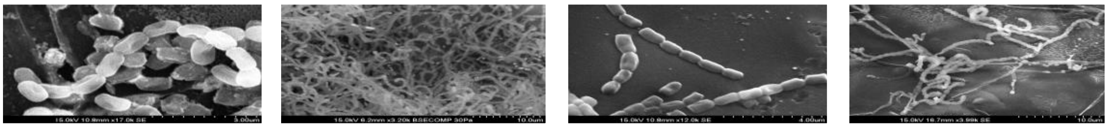
(K13) *S. mutabilis*
(K14) *S. althioticus*
(K16) *S. anandi*
(K17) *S. rochei*



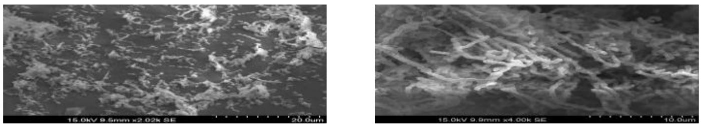
(K20) *S. aurofaciens*
(K21) *S. carpaticus*
(K22) *S. rubrolavendulae*
(K24) *S. viridosporous*



(K26) *S. albogriseolous*
(K28) *S. coelicolor*
(K29) *Actinomycete*
(K30) *S. acrimycini*



(K31) *S. rameus*
(K32) *S. coelicoflavus*
(K33) *S. fradiae*
(K35) *S. coeruleorubidus*



(K37) *S. diasticus*
(K38) *Actinoalloteichus cyanogriseus*

Fig. 4.4. Scanning electron micrographs of the isolates.

Physiological characterisation of the selected strains (28) was done for the determination of optimal growth temperature, pH and ability to utilize ten different carbon sources. In determination of the growth temperature range, it was observed that all the strains could grew between 25˚C and 50˚C (Table 4.4). However, the growth at 30˚C was far more prominent and colonies were harder and compact as compared to the growth at 45˚C and higher temperatures. The isolates also exhibited growth at 50˚C, but none of them was able to grow at temperatures above 50˚C (Table 4.4).

Table 4.4. Growth temperature range of the 28 halotolerant isolates.

Isolate	**Temperature range (˚C)**						
	25	**30**	**35**	**40**	**45**	**50**	**55**
K2	+	+	+	+	+	+	-
K3	+	+	+	+	+	+	-
K4	+	+	+	+	+	+	-
K5	+	+	+	+	+	+	-
K8	+	+	+	+	+	+	-
K9	+	+	+	+	+	+	-
K10	+	+	+	+	+	+	-
K12	+	+	+	+	+	+	-
K13	+	+	+	+	+	+	-
K14	+	+	+	+	+	+	-
K16	+	+	+	+	+	+	-
K17	+	+	+	+	+	+	-
K20	+	+	+	+	+	+	-
K21	+	+	+	+	+	+	-
K22	+	+	+	+	+	+	-
K24	+	+	+	+	+	+	-
K26	+	+	+	+	+	+	-
K28	+	+	+	+	+	+	-
K29	+	+	+	+	+	+	-
K30	+	+	+	+	+	+	-
K31	+	+	+	+	+	+	-
K32	+	+	+	+	+	+	-
K33	+	+	+	+	+	+	-
K34	+	+	+	+	+	+	-
K35	+	+	+	+	+	+	-
K36	+	+	+	+	+	+	-
K37	+	+	+	+	+	+	-
K38	+	+	+	+	+	+	-

+: growth at respective incubation temperature; -: indicates no growth.

The halotolerant isolates were capable of growing at wide pH range of 5.0 to 11.0 (Table 4.5). Although the growth was observed at pH ≤5.0 and also ≥11.0, but it was very slow and insignificant. The pH tolerance of the halotolerant isolates revealed that they were moderately alkali-tolerant exhibiting good growth at pH 7.0 to pH 10.0 (Table 4.5).

Table 4.5. pH tolerance range of the 28 halotolerant isolates

Isolate	**pH range**						
	5.0	**6.0**	**7.0**	**8.0**	**9.0**	**10.0**	**11.0**
K2	+	++	+++	+++	+++	+++	++
K3	+	++	+++	+++	+++	+++	++
K4	+	++	+++	+++	+++	+++	++
K5	+	++	+++	+++	+++	+++	++
K8	+	++	+++	+++	+++	+++	++
K9	+	++	+++	+++	+++	+++	++
K10	+	++	+++	+++	+++	+++	++
K12	+	++	+++	+++	+++	+++	++
K13	+	++	+++	+++	+++	+++	++
K14	+	++	+++	+++	+++	+++	++
K16	+	++	+++	+++	+++	+++	++
K17	+	++	+++	+++	+++	+++	++
K20	+	++	+++	+++	+++	+++	++
K21	+	++	+++	+++	+++	+++	++
K22	+	++	+++	+++	+++	+++	++
K24	+	++	+++	+++	+++	+++	++
K26	+	++	+++	+++	+++	+++	++
K28	+	++	+++	+++	+++	+++	++
K29	+	++	+++	+++	+++	+++	++
K30	+	++	+++	+++	+++	+++	++
K31	+	++	+++	+++	+++	+++	++
K32	+	++	+++	+++	+++	+++	++
K33	+	++	+++	+++	+++	+++	++
K34	+	++	+++	+++	+++	+++	++

K35	+	++	+++	+++	+++	+++	++
K36	+	++	+++	+++	+++	+++	++
K37	+	++	+++	+++	+++	+++	++
K38	+	++	+++	+++	+++	+++	++

+++: good growth; ++: satisfactory growth; +: less than average growth.

For sugar utilisation pattern studies, it was reported that all the 28 halotolerant isolates could grow well on five different sugars, *viz*., D-glucose, xylose, cellobiose, mannose and D-galactose (Table 4.6). Only two isolates, K2 and K35 did not exhibit any growth on D-fructose. Similarly, two isolates, *viz*., K5 and K22 were unable to utilise sucrose as sole carbon source, while isolates K10, K22 and K 34 did not exhibit any growth on L-arabinose. Isolates, K22 and K9 did not utilize raffinose as carbon source. In the case of inositol, only one isolate, K22 exhibited no growth (Table 4.6).

4.3. Biochemical characterisation: Extracellular enzyme production, Hydrogen sulfide production and PGP attribute of the halotolerant isolates

A. Extracellular enzymes production

The 28 halotolerant isolates screened from salt-affected soil produced extracellular hydrolytic enzymes and possessed plant growth promotion (PGP) activities. Qualitative screening of the halotolerant isolates for biochemical attributes like enzymes revealed that 75% of them produced the enzyme amylase and 57.14% produced protease enzyme. In total, 28.57%, 35.71% and 85.71% produced gelatinase, cellulase and urease, respectively. Hydrogen sulfide (H_2S) production was recorded in 39.28% of the isolates (Table 4.7).

On the basis of qualitative assay, twenty one out of twenty eight isolates produced industrially important enzyme amylase as evident from clear zone around the spotted colonies, indicating starch hydrolysis (Fig. 4.5A).

Sixteen and ten isolates produced cell wall degrading enzymes *viz* protease and cellulase (Table 4.7.). Interestingly, in protease assay, the isolates were able to utilize skimmed milk as carbon source within 48-60 h of incubation time (Fig 4.5 B).

Table 4.6. Sugar utilization profile of the 28 halotolerant isolates.

Isolate	Different sugars used as carbon source									
	D-glucose	**D-fructose**	**Xylose**	**Raffinose**	**Cellobiose**	**L-arabinose**	**Mannose**	**D-galactose**	**Inositol**	**Sucrose**
K2	+	-	+	+	+	+	+	+	+	+
K3	+	+	+	+	+	+	+	+	+	+
K4	+	+	+	+	+	+	+	+	+	+
K5	+	+	+	+	+	+	+	+	+	-
K8	+	+	+	+	+	+	+	+	+	+
K9	+	+	+	-	+	+	+	+	+	+
K10	+	+	+	+	+	-	+	+	+	+
K12	+	+	+	+	+	+	+	+	+	+
K13	+	+	+	+	+	+	+	+	+	+
K14	+	+	+	+	+	+	+	+	+	+
K16	+	+	+	+	+	+	+	+	+	+
K17	+	+	+	+	+	+	+	+	+	+
K20	+	+	+	+	+	+	+	+	+	+
K21	+	+	+	+	+	+	+	+	+	+

K22	+	+	+	-	+	-	+	+	-	-
K24	+	+	+	+	+	+	+	+	+	+
K26	+	+	+	+	+	+	+	+	+	+
K28	+	+	+	+	+	+	+	+	+	+
K29	+	+	+	+	+	+	+	+	+	+
K30	+	+	+	+	+	+	+	+	+	+
K31	+	+	+	+	+	+	+	+	+	+
K32	+	+	+	+	+	+	+	+	+	+
K33	+	+	+	+	+	+	+	+	+	+
K34	+	+	+	+	+	-	+	+	+	+
K35	+	-	+	+	+	+	+	+	+	+
K36	+	+	+	+	+	+	+	+	+	+
K37	+	+	+	+	+	+	+	+	+	+
K38	+	+	+	+	+	+	+	+	+	+

+: respective sugar utilized; -: no growth or limited growth on respective sugar as carbon source.

Likewise, twenty four isolates produced the enzyme urease as observed by change in colour of urea degradation from orange media to pink (Fig 4.5 C). Gelatinase activity was observed in eight isolates as evident from liquefaction of gelatin tubes. Cellulase activity was visualized as clear zone produced around spotted colonies of the isolates utilizing carboxymethyl cellulose as carbon source (Fig 4.5 D).

B. Hydrogen sulfide production

Hydrogen sulfide production determines the ability of the microbes to reduce sulfur-containing compounds to sulfides during the process of metabolism. Eleven out of total twenty eight isolates were capable of producing hydrogen sulfide (Table 4.7) as observed from a positive isolate by black colouration on the SIM agar media (Fig 4.5 E).

Plant growth promotory attributes

Isolated strains were studied for their ability to produce plant growth promotory attributes *viz* IAA, phosphate solubilization, siderophore, chitinase, ammonia and HCN production. The PGP activity of the isolates revealed that a total of 42.85% isolates produced siderophore, the iron chelating molecule. 78.57% were ammonia producers while only 21.42% of the isolates were producing HCN. Although phosphate solubilization and chitinase production was detected in 28.57% and 7.14% of the isolates respectively but interestingly, Indole acetic acid (IAA) production was detected in all the 28 halotolerant isolates (Table 4.7).

On the basis of qualitative screening, only two (K17 and K20) of the twenty eight halotolerant isolates were reported to have chitinase activity as seen by a clearing zone around the streaked colony, indicating hydrolysis of chitin (Fig 4.5 F).

Since all the 28 isolates were IAA producers (Table 4.7 and Fig. 4.5 G), the amount of IAA produced was quantified in normal as well as in saline-stressed conditions. Quantitative assay of IAA revealed that increase in salt concentration resulted in abnormal production of the phytohormone by the isolates except two (K34 and

K36) isolates. All the isolates showed increase or decrease in IAA quantity with increasing salt level, without following any regular trend. While in the isolates K34 and K36 a regular increase in the quantity of IAA produced was reported with increase in salt concentration up to 6% (w/v) NaCl level although above 6% level the amount of IAA produced declined. The detailed results are represented in Table 4.8.

Out of the twenty eight halotolerant isolates tested, eight (K9, K13, K17, K28, K29, K31, K34 and K37) isolates showed halo zone formation on the pikovskaya's medium (Fig 4.5 H), confirming their ability to produce organic acids to solubilize the tri-calcium phosphate in the media.

In vitro siderophore production assay revealed that twelve (K2, K8, K9, K12, K13, K17, K20, K28, K29, K34, K36 and K37) isolates were positive as was shown by a bright orange halo zone around the colony on CAS-agar medium (Fig 4.5 I).

Production of HCN and chitinase by the microorganisms is one of the important traits to know the biocontrol potential of the isolates. The organisms producing HCN and chitinase are able to suppress many of the plant pathogenic bacteria and fungi. Out of twenty eight distinct halotolerant isolates, only six (K4, K13, K22, K29, K33 and K35) isolates were able to produce hydrogen cyanide (Table 4.7 and Fig 4.5 J) while only two strains as discussed above were able to produce chitinase.

Ammonia production was observed in twenty two isolates (K2, K4, K5, K8, K9, K10, K12, K14, K17, K20, K21, K22, K26, K28, K29, K30, K31, K32, K34, K35, K36 and K37) (Fig 4.5 K).

Table 4.7. Biochemical characterization of the halotolerant isolates based on extracellular enzyme and hydrogen sulfide production, and plant growth promotion activities.

Isolate	**Extracellular enzyme assay**					**H_2S production**	**Plant growth promotion activities**					
	Amylase	**Urease**	**Gelatinase**	**Protease**	**Cellulase**		**IAA production**	**P-solubilization**	**Siderophore production**	**HCN production**	**Chitinase assay**	**Ammonia production**
K2	+	+	-	+	+	-	+	-	+	-	-	+
K3	-	-	-	+	-	+	+	-	-	-	-	-
K4	-	-	+	-	-	+	+	-	-	+	-	+
K5	+	+	-	-	-	-	+	-	-	-	-	+
K8	+	+	-	+	+	-	+	-	+	-	-	+
K9	+	+	-	-	-	-	+	+	+	-	-	+
K10	+	+	-	-	-	+	+	-	-	-	-	+
K12	+	+	-	+	+	-	+	-	+	-	-	+
K13	+	+	-	+	-	+	+	+	+	+	-	-
K14	-	+	+	+	+	-	+	-	-	-	-	+
K16	+	+	-	+	-	+	+	-	-	-	-	-
K17	+	+	+	-	-	-	+	+	+	-	+	+
K20	+	+	-	-	-	+	+	-	+	-	+	+

K21	+	+	-	+	-	-	+	-	-	-	-	+
K22	-	-	+	+	-	-	+	-	-	+	-	+
K24	+	+	-	+	-	+	+	-	-	-	-	-
K26	+	+	-	-	+	-	+	-	-	-	-	+
K28	+	+	+	+	-	-	+	+	+	-	-	+
K29	+	+	-	-	+	-	+	+	+	+	-	+
K30	+	+	-	-	-	-	+	-	-	-	-	+
K31	+	+	-	-	-	+	+	+	-	-	-	+
K32	-	+	-	-	+	+	+	-	-	-	-	+
K33	+	+	-	+	+	-	+	-	-	+	-	-
K34	+	+	+	+	+	-	+	+	+	-	-	+
K35	-	+	-	+	-	+	+	-	-	+	-	+
K36	+	+	+	+	-	-	+	-	+	-	-	+
K37	+	+	-	-	-	-	+	+	+	-	-	+
K38	-	-	+	+	+	+	+	-	-	-	-	-

+: indicates positive result; -: indicates negative result.

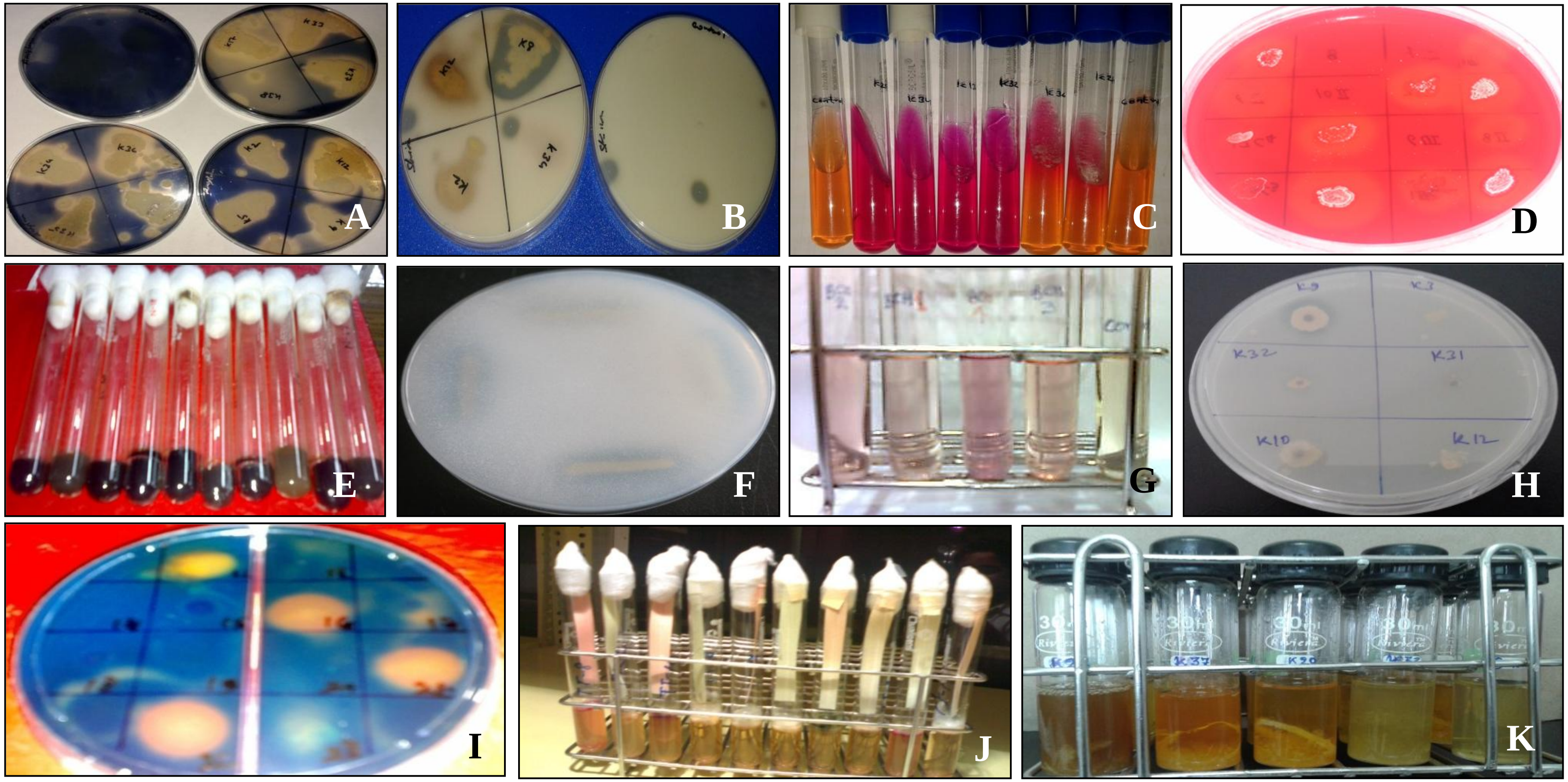


Fig. 4.5. Qualitative assay for extracellular enzyme production and plant growth promotion traits; **(A)** Amylase assay **(B)** Protease assay **(C)** Urease assay **(D)** Cellulase assay **(E)** Hydrogen sulfide production **(F)** Chitinase assay **(G)** IAA Production **(H)** Phosphate solubilization **(I)** Siderophore production **(J)** HCN production **(K)** Ammonia Production.

Table 4.8. Synthesis of IAA (Auxin) by the halotolerant isolates under salt stressed and non-stressed condition.

Isolate	**Concentration of IAA (µg/mg protein) at different NaCl (%) concentrations**				
	0.0	**2.0**	**4.0**	**6.0**	**8.0**
K2	55.10 efg	81.48 c	44.72 cde	57.13 c	35.32 c
K3	23.54 ijk	73.20 c	40.21 def	49.00 cd	15.41 e-h
K4	104.68 b	46.52 d	59.84 c	34.26 c-f	17.74 efg
K5	47.19 fgh	45.54 d	51.45 cd	47.85 cd	21.76 de
K8	14.58 jkl	14.88 efg	7.53 lm	5.93 ef	2.35 j
K9	24.25 ijk	19.55 efg	13.13 klm	15.80 def	6.37 hij
K10	67.78 cd	24.09 e	20.11 g-m	25.66 c-f	8.07 g-j
K12	57.47 def	55.15 d	47.01 cde	20.42 def	13.46 e-i
K13	40.51 h	23.10 e	22.95 g-l	19.02 def	11.16 f-j
K14	17.96 i-l	16.51 efg	17.33 j-m	12.39 ef	7.29 hij
K16	6.68 l	4.09 g	3.21 m	3.06 f	2.48 j
K17	60.70 de	46.56 d	45.48 cde	34.87 c-f	19.66 def
K20	45.97 fgh	46.66 d	35.97 d-h	22.38 def	6.10 hij
K21	27.72 i	25.42 e	18.43 i-m	14.22 ef	8.03 g-j
K22	12.91 kl	6.41 fg	3.73 m	3.66 f	1.96 j
K24	44.39 gh	51.88 d	35.18 d-i	32.01 c-f	19.86 def
K26	26.07 ij	17.23 efg	14.68 klm	12.13 ef	3.59 ij
K28	44.92 gh	27.09 e	14.23 klm	21.57 def	6.02 hij
K29	6.75 l	5.73 fg	4.42 m	3.54 f	1.74 j
K30	10.25 l	3.94 g	5.82 lm	4.56 f	2.20 j
K31	60.27 de	75.58 c	36.60 d-g	22.82 def	9.19 g-j
K32	27.05 i	20.16 ef	10.82 klm	4.99 f	5.99 hij
K33	12.90 kl	4.74 fg	4.58 m	4.88 f	2.68 j
K34	139.11 a	263.55 a	343.93 a	709.91 a	614.53 a
K35	94.04 b	73.31 c	27.05 f-k	39.52 cde	28.34 cd
K36	73.00 c	169.88 b	243.08 b	646.77 b	339.57 b
K37	60.88 de	45.88 d	33.63 e-j	24.87 c-f	19.82 def
K38	76.96 c	22.53 e	19.12 h-m	18.66 def	20.72 def
SEM±	3.81	4.80	5.24	9.82	3.09
CD (*P≤0.05*)	10.79	13.60	14.84	27.83	8.77
CV %	14.4	17.8	20.7	24.9	12

Values are mean of three independent experiments. Means followed by same letter within a row are not significantly different (*P*≤0.05) according to Duncan's Multiple Range Test (DMRT). SEM, standard error of the difference between means; CD, critical difference; CV; coefficient of variance.

Biocontrol activity of the isolates against fungal pathogen

All the isolates were screened *in vitro* by dual culture method against selected plant fungal pathogens [*Sclerotium rolfsii* (SR) and *Macrophomina phaseolina* (MP)] and only two isolates one for SR (K17) and one for MP (K20) gave good antagonists. The isolate K17 gave an inhibition zone of 23.67±0.89 mm against the pathogen SR with inhibition percentage of 57.50 ± 1.67 % (Fig 4.6-I). Similarly in the isolate K20 with an inhibition zone of 27±1.33 mm and percent inhibition of 64.52±1.42% was reported (Fig 4.6-II).

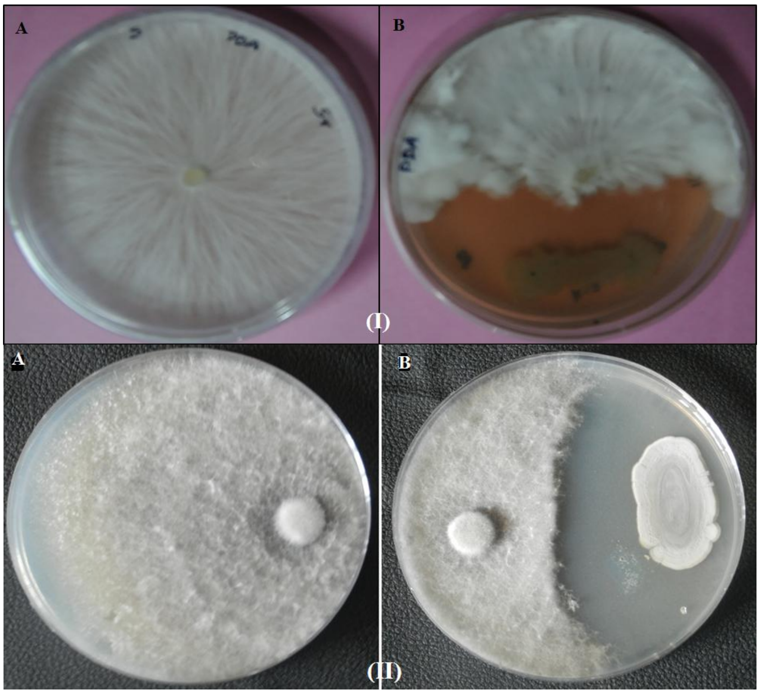


Fig. 4.6. Antifungal activity (dual culture assay) of *Streptomyces* strains (I) K17 against *Sclerotium rolfsii* (II) K20 against *Macrophomina Phaseolina*; A: control; B: dual culture plate.

The microscopic examination of the antagonistic interaction of the isolates K17 with SR and K20 with MP was investigated by scanning electron microscopy. It revealed that the antagonistic isolates reduced apical growth and caused injuries, curling of hyphal tips and distortion of the mycelia in the concerned pathogens (Fig. 4.7 A, B). Degradation of pathogen cell wall was by inter and intra-cellular colonisation of the antagonist that resulted in multiple damaged sites in the infected mycelia.

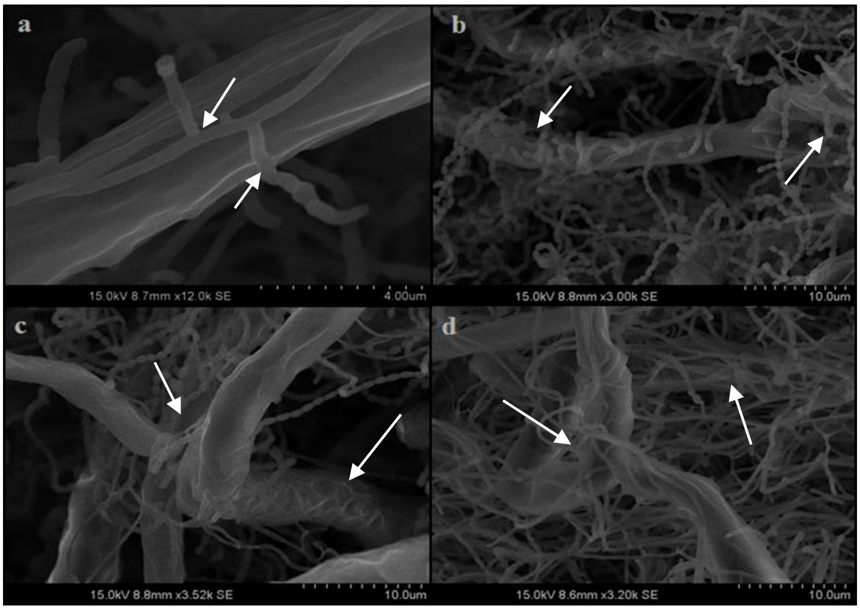


Fig. 4.7 A. Electron micrographs showing morphological changes in fungal mycelia during interaction of SR with K17. **a:** healthy *Sclerotium rolfsii* mycelia infested by actinomycete isolate K17; **b:** proliferation of K17 isolate on fungal mycelia; **c** and **d:** hyphal destruction by antagonistic actinomycetes strain K17. Arrows indicate mycophagy action of antogonist over the intact mycelia and hyphal distortion.

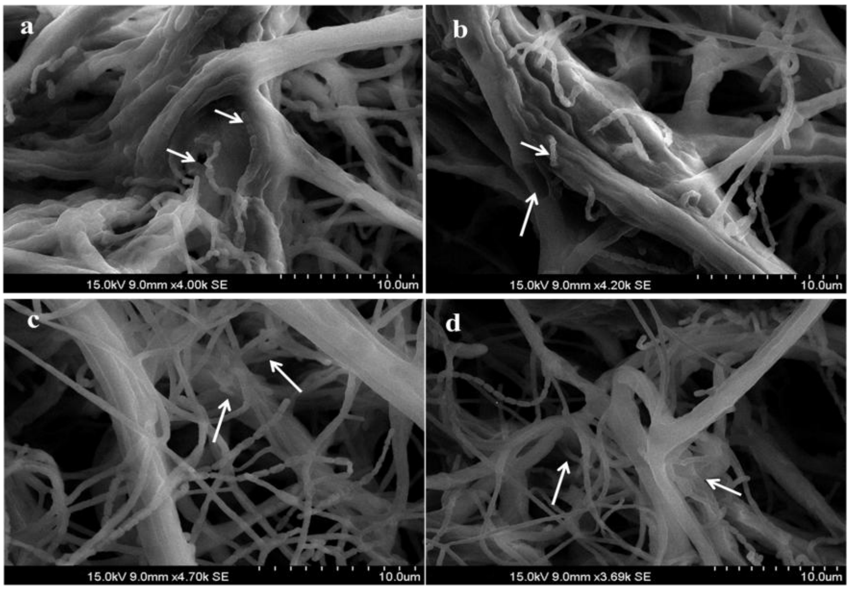


Fig. 4.7 B. Electron micrographs showing morphological changes with fungal mycelia during the interaction of *Macrophomina phaseolina* (MP) with K20. **a:** healthy *M. phaseolina* mycelia infested with actinomycete isolate K20; **b:** proliferation of isolate K20 on the fungal mycelia; **c** and **d:** hyphal destruction by antagonistic actinomycetes strain K20. Arrows indicate mycophagy action of antagonistic strain K20 over the intact mycelia and hyphal distortion.

4.4 Molecular characterization of the halotolerant isolates

i) Isolation and amplification of genomic DNA

Total genomic DNA was isolated from halotolerant potential bacterial isolates with the modified method of Boudjella et al. (2006). The extracted gDNA was agarose gel electrophoresed and digitalized by Gel documentation system. All the isolates yielded a high quality and quantity of genomic DNA (Fig 4.8). The g DNA was quantified and diluted, the stock was stored at 4ºC in refrigerator till further use. The yield of genomic DNA varied from 10 to 120 ng µL^{-1}. The genomic DNA was diluted so as to achieve a concentration of 30-40 ng in 4 µL and was used as a template DNA in PCR reactions.

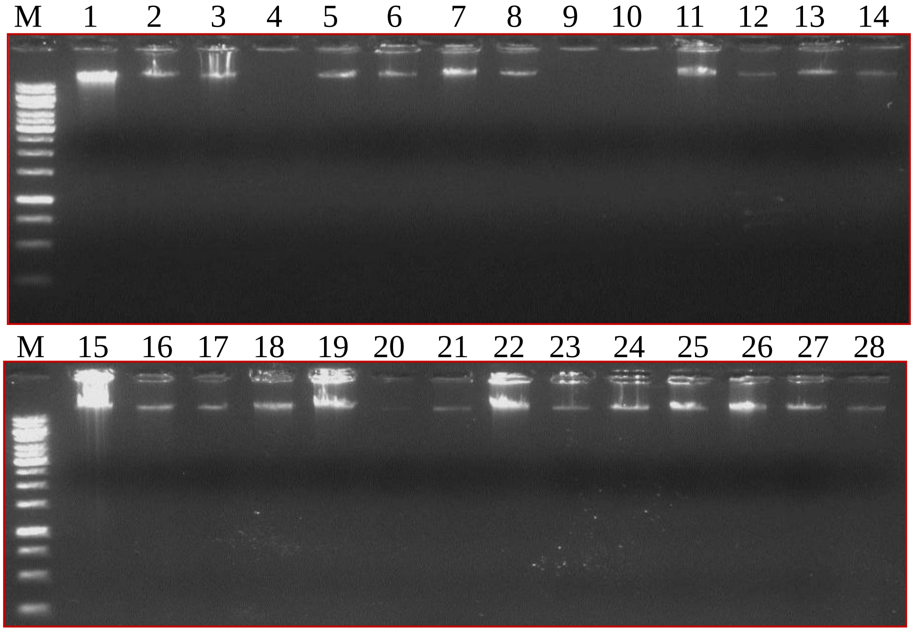


Fig. 4.8. Genomic DNA isolated from the halotolerant actinomycetes isolates. (M represents molecular marker and lane 1-28 represents isolates.

Using the actinomycetes specific universal primer for the PCR, amplification of 16S rRNA gene was performed, and the amplified products were gel electrophoresed and documented successfully. Amplification of 16S ribosomal DNA resulted in a single amplicon of about 1150 bp in all the isolates (Fig 4.9).

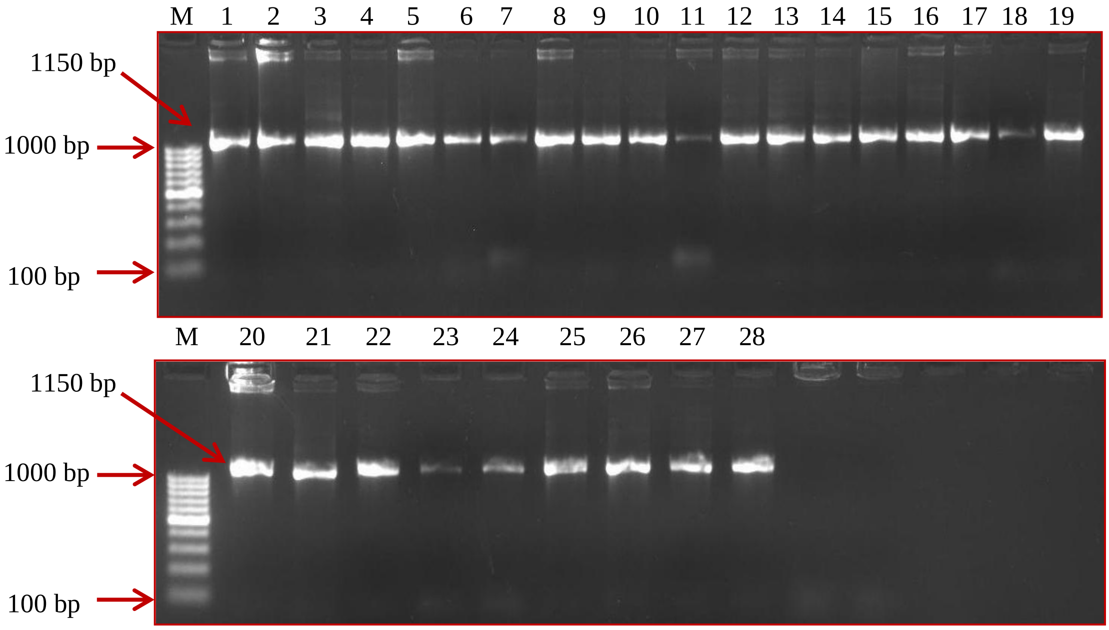


Fig. 4.9. Amplification of 16S rRNA gene from the isolates of actinomycetes using universal primers. Lanes M: 100 bp DNA ladder; 1: K2; 2: K3; 3: K4; 4: K5; 5: K8; 6: K9; 7: K10; 8: K12; 9: K13; 10: K14; 11: K16; 12: K17; 13: K20; 14: K21; 15: K22; 16: K24; 17: K26; 18: K28; 19: K29; 20: K30; 21: K31; 22: K32; 23: K33; 24: K34; 25: K35; 26: K36; 27: K37; 28: K38. The arrow indicates 16S rDNA specific amplified product of 1150 bp.

ii) Restriction Fragment Length Polymorphism (RFLP)

PCR amplification yielded high intensity amplicons which was sufficient for restriction digestion with different tetracutters, *viz*., *TaqI*, *HaeIII* and *MspI.* The characterization of these isolates on the basis of the ARDRA (Amplification of ribosomal DNA by restriction analysis) produced polymorphic patterns with different profiles characterized by 3-4 fragments ranging from 50 bp to 450 bp for different isolates (Fig. 4.10). In ARDRA analysis of twenty eight isolates using restriction enzymes, *Hae*III and *Msp*I were found to be more discriminative compared to *Taq*I. PCR-RFLP was used for construction of phylogenetic tree using Jaccards coefficient of UPGMA of neighbour joining (NJ) method using the software NTSys ver. 2.2.

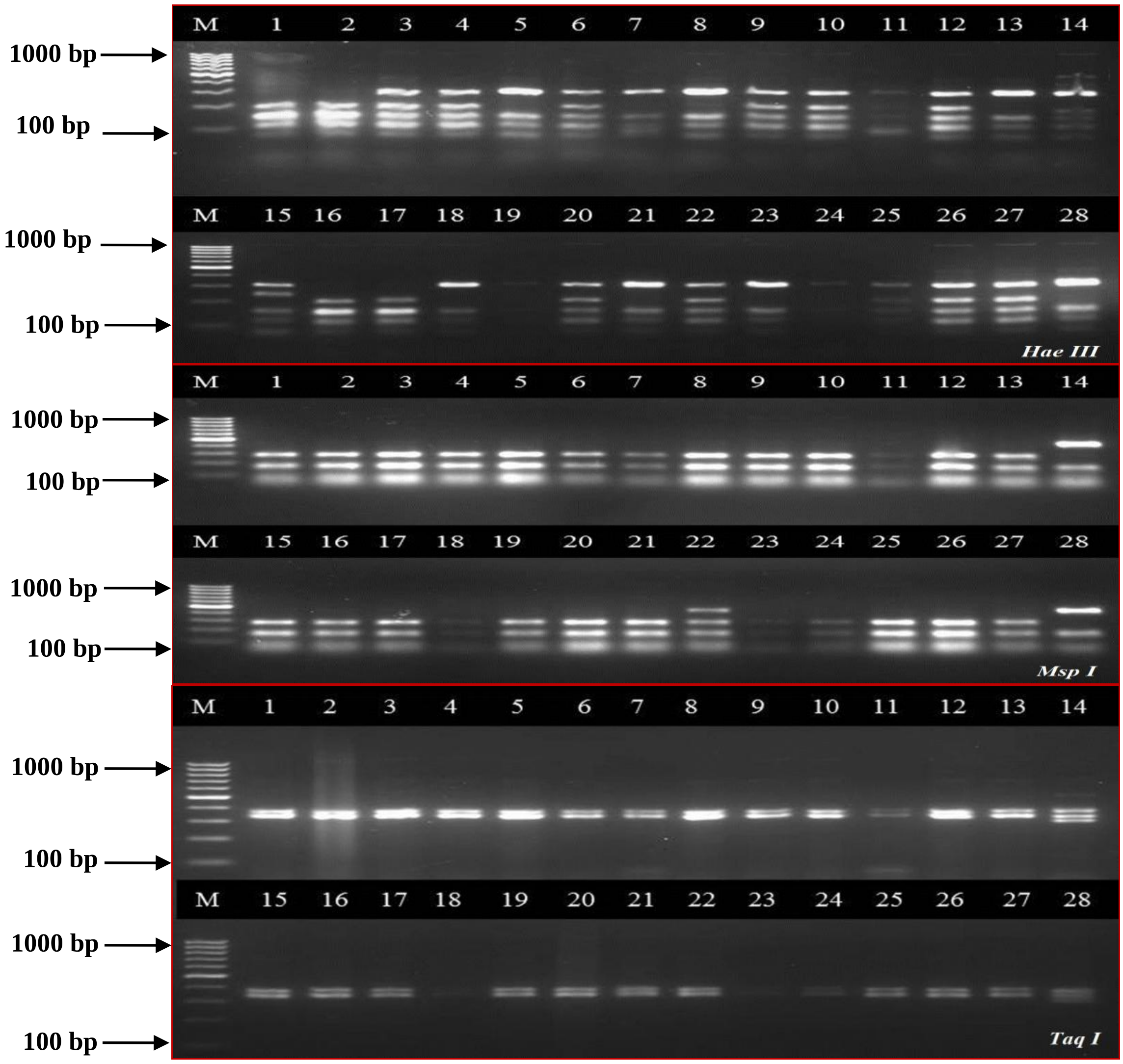


Fig. 4.10. Restriction digestion banding pattern of isolates amplified by 16S rDNA and digested by *Hae* III, *Msp* I and *Taq* I restriction enzymes (Lane M: 100 bp DNA ladder from Bangalore GeNei™ Pvt. Ltd.). 1-K2, 2-K3, 3-K4, 4-K5, 5-K8, 6-K9, 7-K10, 8-K12, 9-K13, 10-K14, 11-K16, 12-K17, 13-K20, 14-K21, 15-K22, 16-K24, 17-K26, 18-K28, 19-K29, 20-K30, 21-K31, 22-K32, 23-K33, 24-K34, 25-K35, 26-K36, 27-K37, 28-K38.

All the isolates were grouped into eight phenons (3 major and 5 minor) in a dendrogram inferred from ARDRA patterns obtained by restriction digestion with three enzymes. Phenon I had 6 isolates, while Phenon II and Phenon IV had 7 isolates each. The Phenons, III, V, VI, VII and VIII had 1, 2, 1, 2 and 2 isolates, respectively (Fig. 4.11).

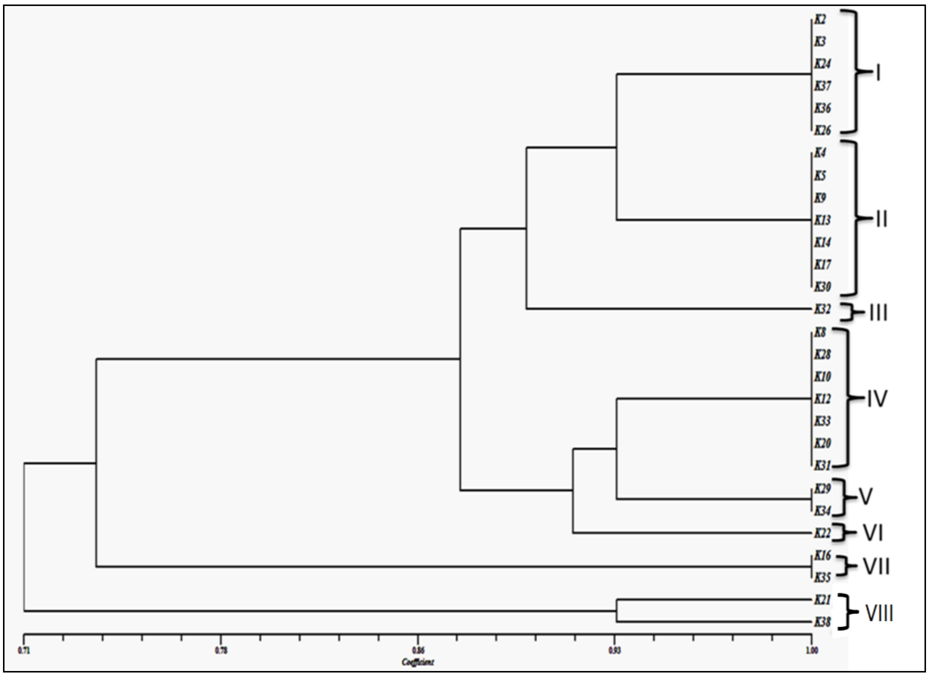


Fig. 4.11. Combined dendrogram of halotolerant actinomycetes isolates based on cluster analysis of ARDRA products with restriction endonucleases *Taq*I, *Hae*III and *Msp*I. Dendrogram is based on the similarity coefficient and clustering was done using un-weighted pair-grouping method based on arithmetic averages (UPGMA) using NTSys software.

iii) Sequencing of 16S rRNA gene, accession numbers, and phylogenetic analysis

The results of 16S rRNA partial gene sequencing of the 28 halotolerant isolates for determining the identity of the isolates with BLASTn revealed a similarity value of more than 98% (Table 4.9) with the strains already present in NCBI database, which indicated that test isolates should be affiliated to following two genera belonging to two families, *viz.*, Streptomycetaceae and Pseudonocardiaceae.

Streptomyces

The most abundant group of isolates were affiliated to the genus *Streptomyces* belonging to Streptomycetaceae family and represented by 26 isolates accounting for 92.85% of the total actinobacterial population.

Actinoalloteichus

The genus *Actinoalloteichus* was represented by a single isolate K38 (3.57% of the total halotolerant isolates). The 16S rRNA sequence analysis of isolate K38 revealed its affiliation to *Actinoalloteichus*. In the phylogenetic tree, the isolate K38 clustered with *Actinoalloteichus cyanogriseus* with 99% sequence similarity (Table 4.9). One isolate was not allotted to any specific actinomycete genera and it was identified as Actinomycete in general. The partial 16S rRNA gene sequences of the 28 strains were submitted to NCBI GenBank with accession numbers as KC511795 to KC511800, KP331747 to KP331752, KP331754 to KP331758, KP331760 to KP331767, KP331769 and KP331771 to KP331772 (Table 4.9).

Table 4.9: Identification of halotolerant actinomycetes isolated from salt-affected soils based on 16S rRNA gene sequence.

S. No.	**Isolate code**	**Identity**	**% Similarity based on BLASTn**	**Accession number**
1	K2	*Streptomyces fradiae*	99	KC511795
2	K3	*Streptomyces fradiae*	99	KP331747
3	K4	*Streptomyces coeruleorubidus*	99	KP331748
4	K5	*Streptomyces thermolilacinus*	99	KP331749
5	K8	*Streptomyces atrovirens*	99	KP331750
6	K9	*Streptomyces gancidicus*	100	KP331751
7	K10	*Streptomyces thermoluteus*	100	KP331752
8	K12	*Streptomyces celluloflavus*	99	KC511796
9	K13	*Streptomyces mutabilis*	100	KP331754
10	K14	*Streptomyces althioticus*	99	KP331755
11	K16	*Strptomyces anandii*	99	KP331756
12	K17	*Streptomyces rochei*	99	KP331757
13	K20	*Streptomyces aureofaciens*	99	KP331758
14	K21	*Streptomyces carpaticus*	99	KC511797
15	K22	*Streptomyces rubrolavendulae*	100	KC511798
16	K24	*Streptomyces viridosporus*	99	KP331760
17	K26	*Streptomyces albogriseolus*	99	KP331761
18	K28	*Streptomyces coelicolor*	99	KP331762
19	K29	*Actinomycete*	99	KP331763
20	K30	*Streptomyces acrimycini*	99	KP331764

21	K31	*Streptomyces rameus*	99	KP331765
22	K32	*Streptomyces coelicoflavus*	99	KP331766
23	K33	*Streptomyces fradiae*	100	KP331767
24	K34	*Streptomyces albogriseolus*	99	KC511799
25	K35	*Streptomyces coeruleorubidus*	99	KP331769
26	K36	*Streptomyces griseorubens*	99	KC511800
27	K37	*Streptomyces diastaticus*	99	KP331771
28	K38	*Actinoalloteichus cyanogriseus*	99	KP331772

A corresponding phylogenetic analysis for 28 isolates of partial 16S rRNA gene sequences was aligned with the retrieved sequences (closest representatives) from NCBI Gene bank databases. The phylogenetic tree was constructed using the software MEGA ver. 4.1 employing neighbour joining (NJ) method. Three major and five minor phenons were formed based on NJ method with 1,000 bootstrap sampling (Fig. 4.12). The isolates, K28, K34, K26, K10, K20, K30, K14, K29, K4, K9 and K8 which showed similarity with *Streptomyces* and Actinomycete were grouped under phenon I. The isolates K16, K17, K13, K37, K35, K32 and K33 showed similarity with different species of *Streptomyces* and were grouped into phenon II. The isolates in phenon III, IV, V, VI and VII were again showing similarity with *Streptomyces* spp. The remaining one isolate K38 was grouped in phenon VIII and showed similarity to rare genera of Actinomycetes and was identified as *Actinoalloteichus cyanogriseus.*

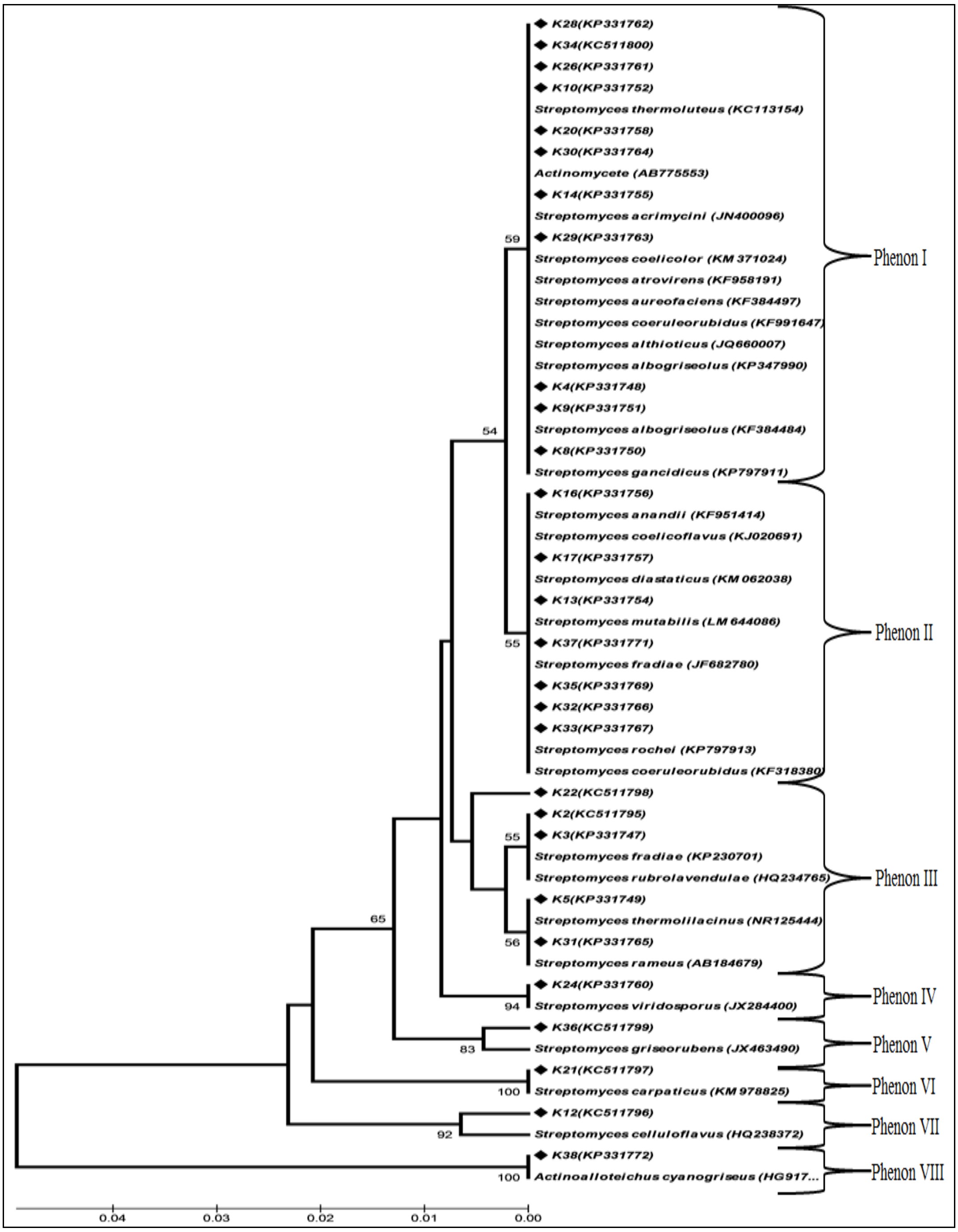


Fig. 4.12. NJ-phylogenetic tree of full 16S rRNA gene sequences from selected isolates. The sequence data for several closely related *Streptomyces* cultures were recovered from GenBank and included in the tree. The boot strap values from 1,000 pseudo-replications are shown at each of the branch points on the tree. Bar indicates % similarity.

PCR amplification of hydrolytic (chitinase) gene

The PCR amplification of hydrolytic gene, chitinase (*chiA*) was performed with the two antagonistic isolates. From the agarose gel image it was clear that the primer amplified ~270 bp product specific to chitinase (*chiA*; Fig. 4.13).

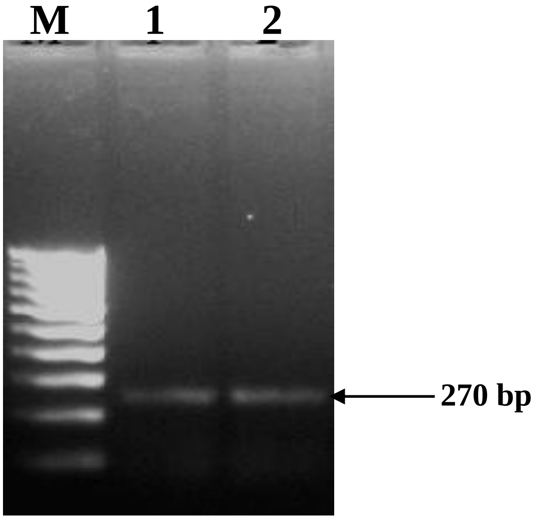


Fig. 4.13. PCR-amplification of chitinase (*chiA*) gene from genomic DNA of the selected isolates of antagonistic *Streptomyces sp.* Lanes M: 100 bp DNA ladder; Lane 1: strain K17; Lane 2: strain K20.

4.5 Proline (Osmolyte/Compatible solute) production studies

Proline accumulation occurs in hyperosmotic (saline) conditions, accounting for its role as osmolyte (compatible solute) in microbes. The biosynthesis of proline by the halotolerant isolates was observed in NaCl (0.0% to 8.0%) amended broth and in absence of NaCl. All the 28 isolates produced proline under normal as well as stressed conditions but a significant increase in proline content was observed in almost all the isolates with increase in osmolarity (Table 4.10). The maximum and continuous accumulation of proline, with increasing salinity stress was reported in isolate K36 followed by isolate K34.

Table 4.10. Production of osmolyte: proline in halotolerant isolates under non-stressed and salt stressed condition.

Isolate	**Proline content (µg/ml) at different concentrations of NaCl (%)**				
	0.0	**2.0**	**4.0**	**6.0**	**8.0**
K2	2.64 f-i	3.13 d-h	5.45 bc	6.57 d	8.40 b
K3	2.58 f-j	3.32 d-h	3.89 e-i	5.41 e	7.19 c
K4	5.81 a	4.41 bc	6.65 a	7.99 abc	2.44 jk
K5	1.54 kl	1.48 jk	3.41 f-j	4.81 efg	6.93 cd
K8	2.70 fgh	2.88 e-i	5.30 bcd	4.10 f-i	4.83 e-h
K9	3.80 de	3.34 d-h	4.01 e-h	4.35 e-h	5.15 efg
K10	3.40 ef	5.31 ab	4.04 e-h	4.59 e-h	2.03 kl
K12	5.26 ab	5.53 a	3.34 f-j	1.74 k	4.76 fgh
K13	3.66 de	3.89 cde	4.12 d-g	2.28 jk	2.54 jk
K14	1.22 l	2.46 hi	2.82 hij	1.19 k	3.71 hi
K16	1.26 l	2.50 hi	2.68 ij	3.14 ij	6.02 de
K17	2.51 g-j	3.13 d-h	3.50 f-j	1.88 k	1.24 l
K20	3.19 efg	4.10 cd	4.78 cde	4.50 e-h	4.88 e-h
K21	1.76 jkl	2.86 f-i	4.86 cde	5.24 ef	4.03 ghi
K22	3.81 de	4.69 abc	6.91 a	7.47 bcd	4.08 ghi
K24	2.15 h-k	2.65 hi	5.07 b-e	7.06 cd	1.48 kl
K26	1.82 i-l	1.98 ijk	2.60 j	2.13 jk	4.45 f-i
K28	3.55 de	4.43 bc	6.20 ab	6.87 cd	6.99 cd
K29	2.16 h-k	2.89 e-i	3.47 f-j	3.79 ghi	2.51 jk
K30	3.98 cde	3.19 d-h	3.92 e-i	3.84 ghi	3.44 ij
K31	1.43 kl	2.36 hij	2.94 g-j	3.17 ij	3.83 hi
K32	3.18 efg	3.76 c-g	4.77 cde	4.92 efg	1.71 kl
K33	3.61 de	4.72 abc	5.01 b-e	5.26 ef	7.31 c
K34	4.34 cd	5.23 ab	5.45 bc	8.19 ab	10.63 a
K35	1.93 h-l	1.17 k	3.24 f-j	3.57 hi	4.88 e-h
K36	4.69 bc	4.76 abc	6.67 a	8.61 a	11.13 a
K37	3.86 de	3.84 c-f	4.29 c-f	4.79 efg	5.59 ef
K38	2.17 h-k	2.76 ghi	3.01 g-j	4.57 e-h	4.83 e-h
SEM±	0.26	0.31	0.38	0.37	0.37
CD (*P≤0.05*)	0.74	0.87	1.06	1.04	1.04
CV %	15	15.4	14.9	13.5	12.9

Values are mean of three independent experiments. Means followed by same letter within a row are not significantly different (*P*≤0.05) according to Duncan's Multiple Range Test (DMRT). SEM: standard error of the difference between means; CD: critical difference; CV: coefficient of variance.

4.6 *In vivo* plant growth promotion assay with wheat (*Triticum aestivum* L.)

i) Selection of actinomycetes strain for *in vivo* assay

Based on salt tolerance capability and multiple PGP attributes, two most promising isolates, viz., *Streptomyces albogriseolus* (K34) and *S. griseorubens* (K36) were selected for *in vivo* pot assay of wheat under non-saline and saline conditions. With increase in salt concentration, an increased amount of IAA, siderophore and proline was observed in both the isolates (K34 and K36). Both the *Streptomyces* isolates synthesized IAA into the medium in fairly good amount (Table 4.11). Isolates K34 and K36 produced 139.11 µg/mg protein and 73.0 µg/mg protein of IAA, respectively. The amount of auxin biosynthesized increased with the increasing concentration of salt in the medium reaching up to the maxima of 709.90 µg/mg and 646.77 µg/mg protein for K34 and K36, respectively (Table 4.11, Fig. 4.14 A).

Table 4.11. Production of siderophore, IAA and proline at different NaCl concentrations by the isolates K34 and K36.

NaCl concentration	K34			K36		
	Siderophore production (%)	IAA production (µg/mg protein)	Proline production (µg/ml)	Siderophore production (%)	IAA production (µg/mg protein)	Proline production (µg/ml)
0%	8.19±0.74^{c}	139.11±12.58^{d}	4.34±0.39^{c}	10.65±0.96c	73.00±6.60d	4.69±0.42c
2%	14.87±1.34^{b}	263.55±23.82^{c}	5.23±0.47^{bc}	12.29±1.11c	169.88±15.36c	4.76±0.43c
4%	28.22±2.55^{a}	343.93±31.09^{b}	5.45±0.49^{b}	25.29±2.29b	243.08±21.97b	6.67±0.60b
6%	31.03±2.80^{a}	709.90±64.17^{a}	8.19±0.74^{a}	29.39±2.66a	646.77±58.47a	8.61±0.78a

Mean±SD value in the same column followed by the same letter(s) are not significantly different ($P<0.05$) according to DMRT. For each experiment, values represent the means of three independent experiments.

Similarly, siderophore production also increased subsequently in the presence of salt (NaCl). Isolate K34 produced 8.19% siderophore in broth without NaCl which increased to 31.03% in 6% NaCl amended broth. Similar results were observed with isolate K36 in which the siderophore production increased from 10.65% in the absence of NaCl to 29.39% in 6% NaCl amended broth (Table 4.11, Fig. 4.14 B).

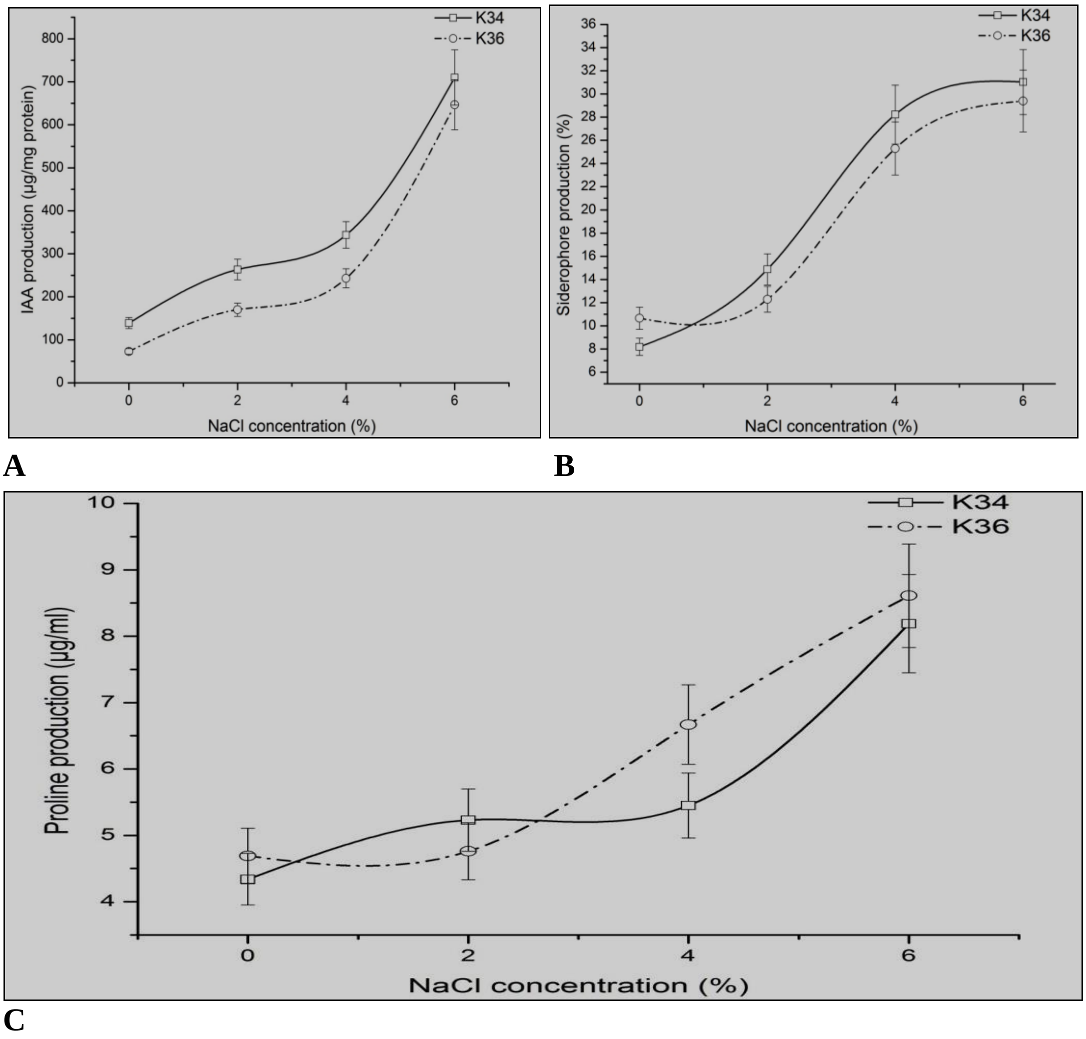


Figure 4.14. Effect of sodium chloride (NaCl) concentration on production of metabolites by isolates K34 and K36 (A) IAA, (B) Siderophore, (C) Proline.

The biosynthesis of proline by both the isolates was observed in broth amended with and without NaCl but the proline concentration increased with increasing salt (NaCl) concentration. Isolate K34 synthesized 4.34 µg/ml of proline and isolate K36 produced 4.69 µg/ml in absence of NaCl, which increased with increasing concentration of NaCl. And the maximum proline production was observed at 6% of NaCl with 8.19 µg/ml and 8.61 µg/ml in K34 and K36, respectively (Table 4.11, Fig. 4.14 C).

ii) *In vivo* assay in non-saline conditions

In the case of sterilized wheat seeds, 15 days after sowing of in non-saline conditions, it was observed that the treatments with isolate K34 (T1), K36 (T2) and a combination of both K34+K36 (T3) resulted to an increase in the vegetative plant growth parameters such as shoot length and root length. Test plant grown in non-saline conditions showed a significant increase in shoot length and root length in treatments T1, T2 and T3 with the maximum increase of 58.7% in shoot length and 102.8% in root length, respectively in T3 as compared to control (Table 4.12, Fig. 4.15 A, B). The concentration of proline also increased in the plants treated with bioformulation as compared to plants in control i.e. without treatment. Maximum concentration of proline was reported in plants having treatment T2 followed by T3 and T1 (Table 4.12).

Table 4.12. Effect of actinomycetes based bioformulation treatment on the seedling growth and proline concentration of wheat seedlings after 15 days after been sown in plastic cups in non-saline condition.

Treatment	**Shoot length (cm)**	**Root length (cm)**	**Proline (µg/mg dry wt.)**
Control	18.37 d	7.43 c	1.73 b
T1 (K34)	22.33 c	10.37 b	2.14 b
T2 (K36)	25.63 b	12.20 b	2.86 a
T3 (K34+K36)	29.17 a	15.07 a	2.64 a
SEM±	0.24	0.75	0.14
CD (p=0.05)	0.84	2.59	0.50
CV (%)	1.8	11.5	10.7

Values are mean of three independent experiments. Means followed by same letter within a row are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test (DMRT). SEM, standard error of the difference between means; CD, critical difference; CV; coefficient of variance.

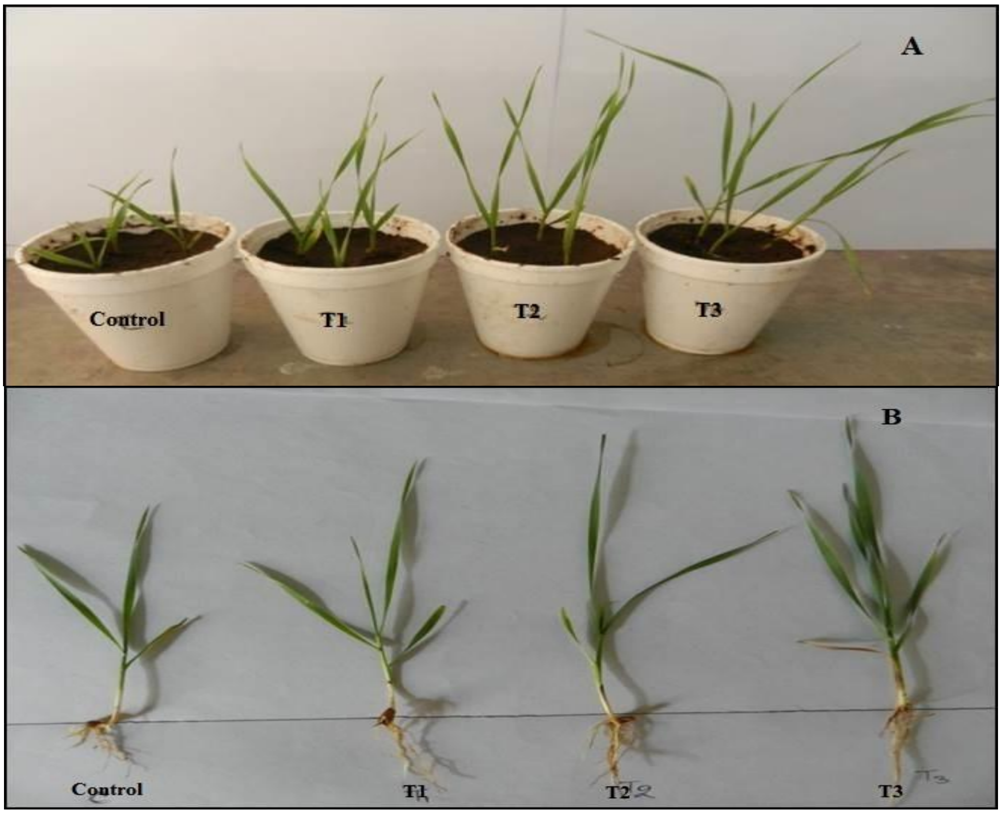


Fig. 4.15. Effect of the treatment of actinomycetes isolates on growth of 15 days old wheat plants under non-saline condition.

iii) Pot study under saline conditions

In saline condition a significant decrease in shoot length and root length in wheat plant was observed as compared to non-saline condition in control as well as treatments T1, T2 and T3. But the soil in which treatment with the actinomycetes isolates was done the adverse effect of salinity was alleviated as evident from shoot length and root length as compared to control and also the proline content was better (Fig 4.17). The data recorded after 15 DAS and 30 DAS clearly showed a marked increase in shoot length and root length in the soil treated with *Streptomyces* isolates and also the proline content in the plants was more in actinomycete treated soil than in control. Although the increase in shoot length, root length and proline content was reported with both the isolates individually (Treatment T1-K34 & T2-K36) but the result of treatment in which both the isolates (T3-K34+K36) were used in combination was better (Table 4.13 & Fig 4.16).

Table 4.13. Effect of the application of *Streptomyces* isolates on growth characteristics of wheat plants grown in saline conditions.

Treatment	**15 days after sowing**			**30 days after sowing**		
	Shoot length (cm)	**Root length (cm)**	**Proline (µg/mg dry wt.)**	**Shoot length (cm)**	**Root length (cm)**	**Proline (µg/mg dry wt.)**
Control	10.93 c	4.23 c	1.65 c	18.43 d	8.57 c	1.94 c
T1 (K34)	16.57 b	9.13 b	4.02 b	32.80 c	18.20 b	4.17 b
T2 (K36)	15.63 b	8.93 b	3.94 b	36.67 b	16.87 b	4.08 b
T3 (K34+K36)	22.03 a	14.23 a	4.82 a	39.37 a	22.27 a	5.04 a
SEM±	0.55	0.46	0.13	0.64	0.95	0.16
CD (p=0.05)	1.91	1.61	0.44	2.20	3.29	0.55
CV (%)	5.9	8.8	6.1	3.5	10	7.2

Values are mean of three independent experiments. Means followed by same letter within a row are not significantly different ($P \leq 0.05$) according to Duncan’s Multiple Range Test (DMRT). SEM, standard error of the difference between means; CD, critical difference; CV; coefficient of variance.

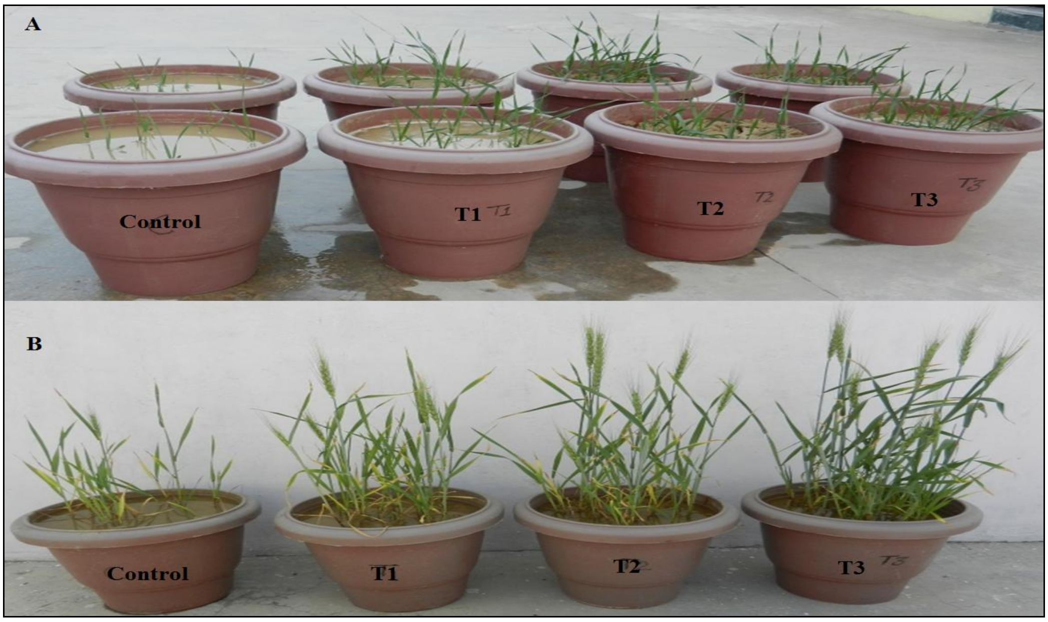


Fig. 4.16. Wheat co-inoculated with actinomycete isolates K34 and K36 alone and in combination. **A:** during early stages; **B:** at the time of flowering and towards maturity.

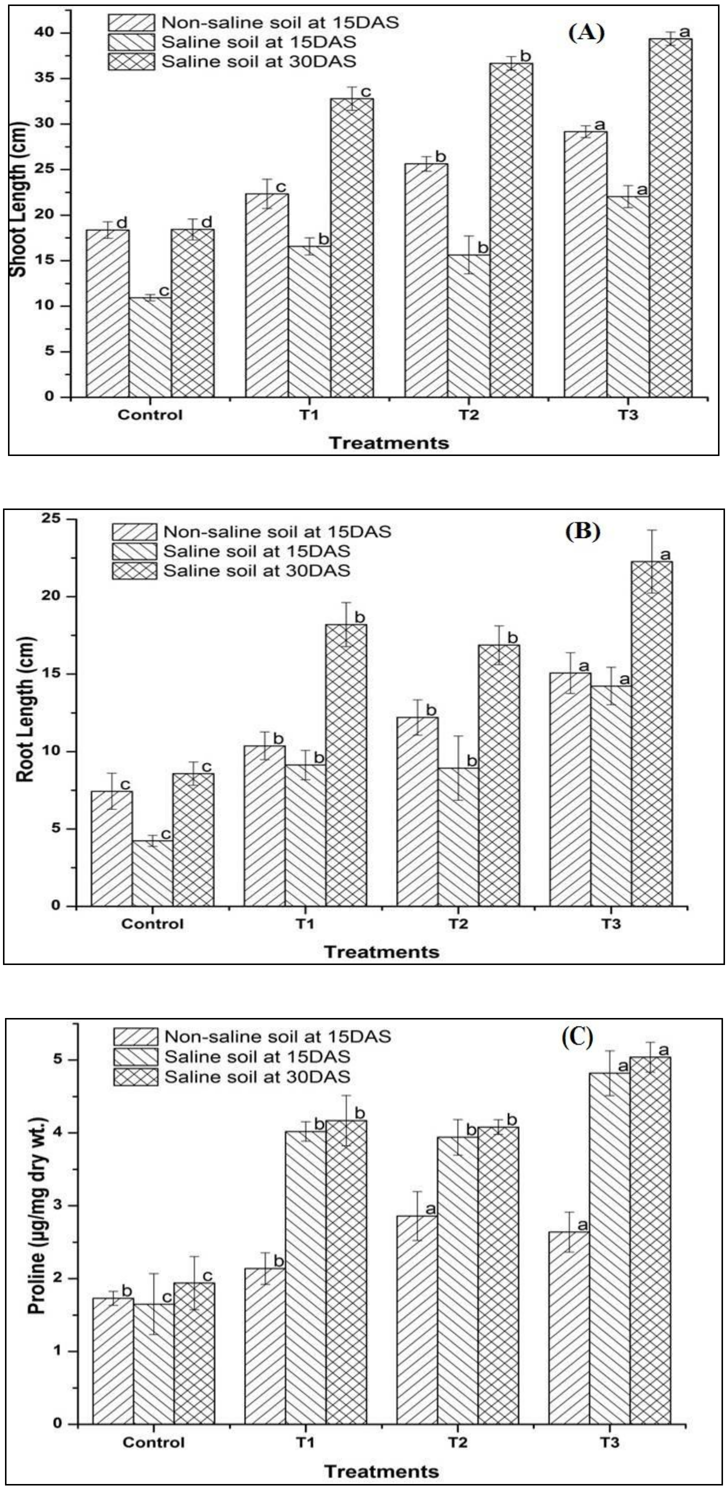


Fig. 4.17. Comparative graph for effect of actinomycete isolate treatments and control on (A) shoot length, (B) root length, and (C) proline accumulation in wheat under saline and non-saline condition.

iv) Effect of the actinomycete isolates on yield parameters of wheat plant in saline conditions under pot assay

Application of the potent halotolerant actinomycete strains significantly increased plant yield in combination as well as individual inoculation treatments as compared to uninoculated control (Table 4.14, Fig 4.18). Maximum number of tiller per plant was recorded in treatment T3 (combination of both strains K34 and K36) followed by T1 (K34).

Table 4.14. Effect of actinomycete strains co-inoculation on yield parameters of wheat under saline condition in pot experiment.

Treatment	**No. of tillers/plant**	**No. of spikes/plant**	**Spike length (cm)**	**No. of spikelets /spike**	**Grain yield (g/plant)**	**Straw yield (g/plant)**
Control	0.67 b	1.00 a	5.70 b	7.33 b	2.34 c	3.89 c
T1 (K34)	2.33 a	2.00 a	8.80 a	13.00 a	4.01 b	6.18 ab
T2 (K36)	2.00 ab	2.00 a	8.47 a	13.33 a	3.97 b	5.70 b
T3 (K34+K36)	2.67 a	2.33 a	9.80 a	14.00 a	4.90 a	6.71 a
SEM±	0.46	0.47	0.37	0.42	0.15	0.20
CD (p=0.05)	1.60	1.63	1.29	1.45	0.52	0.70
CV (%)	41.7	44.5	7.9	6.1	6.8	6.2

Values are mean of three independent experiments. Means followed by same letter within a row are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test (DMRT). SEM, standard error of the difference between means; CD, critical difference; CV; coefficient of variance.

Likewise, in other parameters *viz.* number of spikes/plant, spike length and number of spikelets/spike also, the maximum increase was observed in treatment T3. Both the strains caused increase in grain yield and straw yield when used individually as well as in combination. Maximum enhancement in grain yield per plant (109.4%) and straw yield per plant (72.5%) was recorded with treatment T3 in which both the strains were used in combination as compared to uninoculated control followed by T1 (grain yield: 71.4%, straw yield: 58.9%) and T2 (grain yield: 69.7%, straw yield: 72.5%).

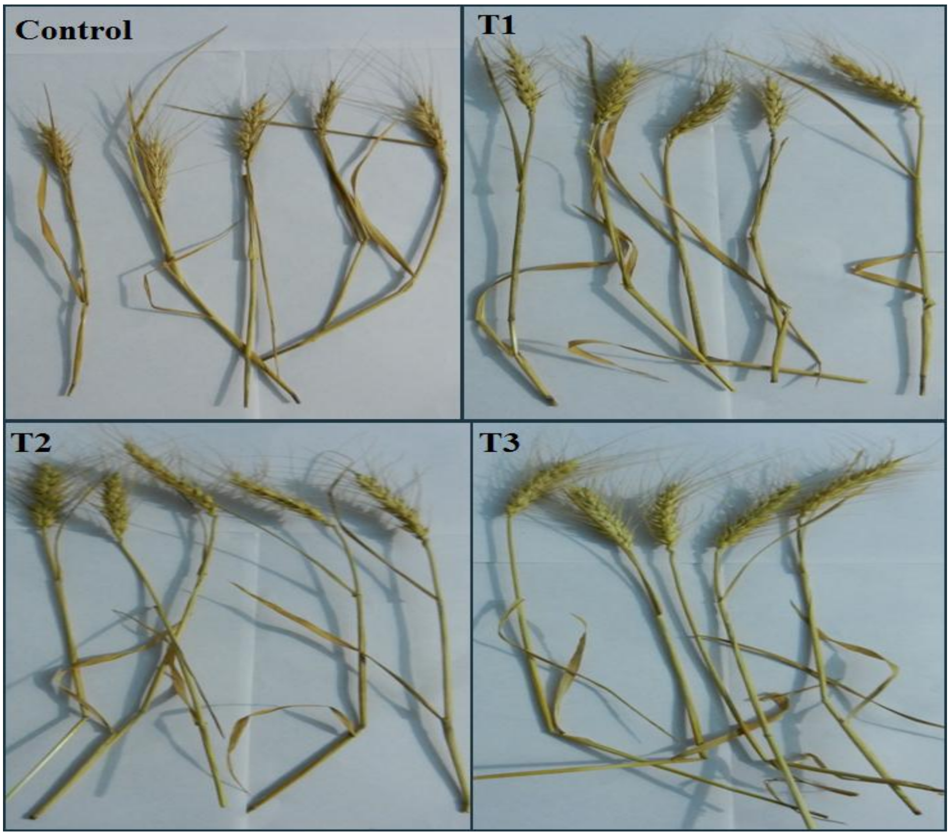


Fig. 4.18. Wheat after harvest.

The data recorded for vegetative parameters in wheat under saline and non-saline conditions, as well as the yield parameters in saline condition clearly indicated that the treatment (T3) in which, consortium of halotolerant *Streptomyces* strains were used, gave better results as compared to control and individual strains. This consortium of halotolerant *Streptomyces* could be utilized as potential bioinoculant under salt stressed condition which will help the crop to overcome the negative impact of salinity.

Chapter 5

DISCUSSION

Chapter 5

Discussion

Indo-Gangetic plains is one of the most fertile and productive regions of India, but reckless application of chemical fertilizers and irrigation with surface saline water has led to deterioration of soil quality and health as well as increase in salinity in this region (Sharma et al. 2015). The floodplain ecosystem of Uttar Pradesh offers a unique opportunity in the evaluation of salt-tolerant microbial diversity for improved and sustainable crop productivity. Actinomycetes population is such microbial population that has been identified as one of the important and prominent group of soil microbes which differ with soil type and pH, geographical location and climatic conditions (Arifuzzaman et al. 2010). The present study offers an overview on molecular phylogeny, salt tolerance, plant growth promotion activity and saline stress remediation potential of halotolerant actinomycetes isolated from the saline soil zones of IGP, India.

Physico-chemical characteristics from this study confirmed that the soils of IGP region of Kanpur, Auraiya, Etawah, Fatehpur and Mainpuri districts are affected with salinity and sodicity and are low in nutrient status as indicated by low phosphorus, organic carbon and nitrogen content in these soils. Similar results have been reported for some places of IGP accounting for the saline sodic status of the soil (Malviya et al. 2011, Sharma et al. 2015). The saline soil environments have recurrently been explored as a source of actinomycetes with important enzymes and secondary metabolites as well as diversity of actinobacteria is extensively studied in such habitats (Hamedi et al. 2013; Saker et al. 2015). Hence, deciphering the molecular phylogeny of actinobacteria inhabiting saline soil zone of IGP, India, is highly desirable. In the present study, the selective isolation methods were used to acquire actinobacteria which succeeded with isolation of both *Streptomyces* (common and predominant actinobacteria) and non-*Streptomyces* (rare actinobacteria), *viz.*, *Actinoalloteichus*. Although, the genera *Streptomyces* was predominant, with the maximum number of isolates (26 out of 28). Similarly selective processing methods and isolation media were used to isolate diverse actinobacteria from saline habitats (Jensen et al. 2005; Malviya et al. 2011, 2014). Salinity tolerance screening of isolated actinobacterial isolates was done and it was reported that out of 55 isolated strains, 28 actinobacterial strains were halotolerant, with optimum growth at 8% NaCl. This salt range clearly indicated that the isolates belonged to intermediate salt tolerant group. These results are in accordance with the findings of Malviya et al. (2011) who reported the salt tolerance capability of *Streptomyces* isolated from IGP region to be up to 8% NaCl. The ability of *Streptomyces* to tolerate high concentrations of NaCl is well known (Waksman 1959). In studies performed by Sharma et al. (2015) on diversity of salt tolerant *Bacilli* from IGP regions it was found that the bacterial isolates were tolerating variable salt concentrations (6-14%). Likewise, Shrivastava et al. (2015) reported halotolerant *Streptomyces* from Mangrove ecosystem with salt tolerance limit of 8% and growth optima at 6% NaCl.

Halophilic *Streptomyces* having potential to be utilized in agricultural fields have previously been reported from India by Vasavada et al. (2006). However, the ability of salt tolerance of beneficial microorganisms is essential for their survival in saline soils, but is not enough to ensure plant improvement under salt stress. In studies by Aly et al. (2003), the negative impact of salinity on number of stested PGPRs resulted in an insufficient salt tolerance of maize.

Plant growth promotion activity is one of the important attributes responsible for salinity stress remediation in plants. To characterize any rhizobacteria as PGPR, it should possess attributes like production of diverse metabolites including indole acetic acid (IAA), siderophores, ammonia, and activities such as phosphate solubilization (El-Deeb et al. 2012; Goswami et al. 2014). The efficacy of actinomycete isolates for PGP has previously been reported by Nassar et al. (2003), El-Tarabily (2008) and Gopalakrishnan et al. (2012).

As far as plant growth promoting attributes of the isolates is concerned IAA production was the most prominent trait observed in all the isolates. This phytohormone has been implicated in increasing root growth, resulting in greater root length and surface area, which enables the plant to access more nutrients from the soil. In the present study, 100% of the halotolerant actinobacterial isolates were

IAA producers but the concentration of IAA varied. The IAA production was negatively affected by increasing salt concentration in all the isolates except the isolates K34 and K36 which showed an increase in amount of IAA with increasing concentration of NaCl (upto 6% NaCl level). Similar results were also reported by Gopalakrishnan et al. (2014) in a study on six actinobacteria, isolated from herbal vermicomposts. It is reported that ~80% of microorganisms isolated from the rhizosphere of crops possess the ability to synthesize and release auxins as secondary metabolites which are known to promote root elongation and plant growth (Patten and Glick 2002). On the similar lines production of IAA was positively affected by increased salinity in *Streptomyces* isolate C isolated from wheat field in Iran (Sadeghi et al. 2012). The range of IAA produced was 4.7 and 2.4 µg/ml in saline (300mM) and non-saline conditions, respectively. In the present study, production of luxuriant amount of IAA in saline (8% NaCl) and non-saline conditions in both the isolates K34 (614.53 and 139.11 µg/mg protein) and K36 (73.0 and 339.57 µg/mg protein) was recorded. By producing the plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial for their growth.

Phosphate solubilisation is one very important traits of plant growth promotion as microorganisms solubilize insoluble phosphate thus making it available to plants. Several bacterial and fungal species are reported to be phosphate solubilisers (Rashid et al. 2004). Although, there are few reports on phosphate solubilisation by *Streptomyces*. In the current study, eight of the twenty eight halotolerant isolates tested were found to be phosphate solubilisers. Gupta et al. (2010) and Sadeghi et al. (2012) also reported phosphate solubilisation by *Streptomyces* isolate. In a study on halotolerant *Streptomyces* isolates from mangrove ecosystem, Shrivastava et al. (2015) have reported phosphate solubilisation by 50% (4 out of 8) of the isolates.

Iron, an essential element of life is unavailable to plants and microbes in an aerobic environment due to its principal insoluble form as Fe^{3+}. Siderophores, low molecular iron chelators, produced by various soil microorganisms bind Fe^{3+} and makes it available for its own growth and also for plants (Wang et al. 1993). There are several reports that show microbial siderophores have a positive correlation with plant growth promotion and siderophores being one of the key factors that should be considered in PGPR screening programs (Wang et al. 1993; Tokala et al. 2002; Goswami et al 2014a). *Streptomyces* species are well known for the production of hydroxamate type of siderophores, which can inhibit phytopathogen growth by competing for iron in rhizospheric soil (Khamna et al. 2009). Tan et al. (2006) suggested that the production of siderophore is an important factor for phytopathogen antagonism and developing growth of the plant. In the present investigation, of the 28 halotolerant isolates, 12 were recorded to be siderophore producers and two isolates, K34 and K36 were able to chelate iron even under saline conditions. These results are in accordance with the reports of Sadeghi et al. (2012) where a positive effect of salinity on siderophore biosynthesis in *Streptomyces* isolate was observed. In contrast, Aragonda et al. (2010) have reported a negative impact of salinity on siderophore production in bacterium *Chromohalobacter salexigens*.

Ammonia production is another important PGP trait, where an organism can break down complex nitrogenous materials like peptones to release ammonia in soil which is taken up by plant as a nutrient source. Accumulation of ammonia can occur in nitrogen rich soil creating an alkaline condition responsible for suppressing the growth of certain fungi (Jha et al. 2012). In the present study, most of the isolates (22 out of 28) were ammonia producers; the result was in parity with the studies done by Passari et al. (2015) on endophytic actinomycetes.

Chitinase from microorganisms is crucial for the degradation and recycling of carbon and nitrogen trapped in insoluble chitin (Kim-Chi et al. 2011) and is widely used for the preparation of biopesticides. The results of present investigation showed that two halotolerant actinomycetes isolates were positive for extracellular chitinase production. These results were consistent with the reports of Taechowisan et al. (2003), who reported 4.56% isolates to possess chitinase gene and Passari et al (2015), who demonstrated 86.3% isolates with chitinase activity in endophytic actinomycetes. Similarly, a halotolerant *Streptomyces* isolate with chitinase activity was reported by Yandigeri et al. (2015) from Chilika Lake, India.

HCN plays an important role in disease suppression (Wei et al 1991); in this study, six of the 28 isolates (21.42%) were recorded to be positive for HCN production, and most of which belong to *Streptomyces sp*. Patil et al. (2010) reported *Streptomyces toxytricini* strain vh22 as HCN producer. Malviya et al. (2011) also found that some of the *Streptomyces* isolates from IGP, India exhibited capability to produce HCN.

It is well known that *Streptomyces* species are capable of inhibiting pathogens by producing extracellular cell wall degrading enzymes (Houssam 2009; Malviya et al. 2014). While many actinomycetes show a relatively broad spectrum of biological activity, it is important to emphasise that such activity may be due to multiple compound secreted by actinomycetes rather than a single inhibitory compound. In the present investigation, the halotolerant actinomycetes were screened for antagonistic activity against two selected fungal phytopathogens *viz*., *Sclerotium rolfsii* (SR) and *Macrophomina phaseolina* (MP). Interestingly only two isolates: K17 against *S. rolfsii* and K20 against *M. phaseolina* were found to be potential antagonists as revealed from dual culture assay. Both the isolates belonged to *Streptomyces* genera and were identified as *Streptomyces rochei* (K17) *and S. aureus* (K20). There are various mechanisms involved in the disease suppression, one of the primary mechanism of pathogen inhibition as used by plant growth promoting rhizobacteria (PGPR) includes the production of antibiotics, lytic enzymes, volatile compounds and siderophore (Harikrishnan et al. 2014).

The mode of action of *Streptomyces* strain K17 and K20 on phytopathogens analyzed by SEM showed gradual destruction of hyphae leading to death due to cytoplasmic extrusion. This observation once again validates their potential to inhibit pathogens, although the exact mode of action of the strains is under investigation but such inhibitory activities could be attributed to the presence of enzyme chitinase and ability to produce siderophore. The ability of *Streptomyces* isolates to act as potential antagonists is reported and well documented. Khamna et al. (2009) reported inhibition of *Sclerotium rolfsii* by *Streptomyces* species. In a study by Gopalakrishnan et al. (2011) it was reported that out of 137 actinomycetes isolated from herbal vermicompost, 8 isolates were potential antagonist against *M.*

phaseolina with PGP attributes and were belonging to *Streptomyces* genera which is in accordance with the findings of the present study. Al-Askar et al. (2013) reported *Streptomyces tendae* RDS16 to be strongly antagonistic against three fungal pathogens *viz*., *Fusarium oxysporum*, *Macrophomina phaseolina* and *Sclerotium rolfsii* which again supports the fact that *Streptomyces* genus constitutes species with good antagonistic activity, having potential to produce the antifungal compounds.

Characterization of the isolates is not only important to understand the role of these microbes in the particular niche but also to understand the requirement and conditions for their survivability in the particular specific niches (Ramesh and Mathivanan 2009). In the present study, identification of the 28 halotolerant isolates was done by polyphasic taxonomy including morphological physiological and molecular characterization. The results found were very diverse as most of the isolates were grey aerial spore bearers (17 isolates), followed by pink aerial spore (4 isolates), cream spore mass (3 isolates), white aerial spores (2 isolates) and one each had ivory and ash brown aerial spores. Likewise, diversity was observed in substrate mycelial morphology with different colours in which yellow and its variant was most prevalent. Soluble pigment formation was recorded in only five isolates. In fact, the species of same genera, *Streptomyces* vary significantly in morphological characteristics. These results are in accordance with previous studies on actinomycetes (Jose and Jebakumar 2012; Malviya et al. 2014). The morphological appearance of isolates compelled to tentatively identify them, as belonging to genus *Streptomyces*. It is the predominant genera of actinobacteria isolated from various ecological niches (Malviya et al. 2011, 2014; Jose and Jebakumar, 2012).

Many species of *Streptomyces* were reported to grow over a restricted pH range with pH optima of 7.0, but little attention has been given to alkali-halotolerant actinomycetes (Babu and Goodfellow 2008; Hozzein et al. 2011). In present study the halotolerant isolates were reported to tolerate a wide range of pH establishing their alkali-tolerant behaviour. These findings are in accordance with the reports of

Malviya et al. (2014) who studied the alkali-halophilic actinomycetes from Chilika brackish water lake, India.

For deciphering the phylogenetic affiliation of the isolated halotolerant actinobacterial isolates, highly recognised phylogenetic marker, 16S rRNA gene was studied (Ludwig and Schleifer 1994). In the present study, ARDRA was employed to assist in differentiation among the taxonomic groups. ARDRA is an extension of RFLP technique; which employs the tetra-cutter restriction enzymes to discriminate the microbes at inter and intra-genus levels (Cook and Meyers 2003; Ahmad et al. 2011). In the current study, a set of three restriction endonucleases *Msp* I, *Hae* III and *Taq* I that specifically recognize the sequence 'CCGG', 'GGCC' and 'TCGA' respectively were used. The results of the different RFLP patterns obtained allowed to effectively differentiate the strains into distinct groups of actinobacteria. Phylogenetic analysis of 16S rRNA gene sequences of the isolates suggested that the obtained actinobacterial population is composed of *Streptomyces* and *Actinoalloteichus*. *Streptomyces* was found to be the predominant genus among the isolated actinobacterial genus from saline soil zones of IGP, India. The predominance of *Streptomyces* genera is also reported in other saline environments (Kushner 1978; Satheeja and Jebakumar 2011; Jose and Jebakumar 2013). Apart from *Streptomyces* genera, one isolate from rare genera *Actinoalloteichus* was also isolated. Currently, this genus comprises four validly described members which include *Actinoalloteichus cyanogriseus* isolated from a soil samples collected from the Yunnan province of China (Tamura et al. 2000), *A. spitiensis* isolated from cold desert of the Indian Himalayas (Singla et al. 2005), *A. hymeniacidonis* isolated from marine sponge (Zhang et al. 2006) and *A. nanshanensis* isolated from the rhizosphere of a fig tree (Xiang et al. 2011). In the present study, the existence of member of rare genus *Actinoalloteichus* in saline soil of IGP from selected regions has been reported for the first time.

Interestingly, some of the isolates possessed similar morphological characters and also grouped closely in ARDRA generated dendrogram. However, in the NJ dendrogram, based on 16S rRNA gene sequences, they were found to fall under different clusters. This may be either due to the genetic variation of these strains or may be due to mutation or other genetic changes, like recombination (Vardhan et al. 2011; Malviya et al 2014).

The phenotypic and biochemical characteristics of strains provided interesting and basic information for screening of morphotypes. Compared to previous reports the heterogeneity and diversity of the isolated strains in this study was recorded to be very low (Maviya et al. 2011, 2014; Jose and Jebakumar 2012, 2013). This might be due to the prominent role of salinity on microbial selection process. Earlier reports also indicate that beneficial microbes isolated from a saline environment are more likely to survive inhibitory salt concentrations than their counterparts from non-saline habitats (Hua et al. 1982; Siddikee et al. 2011). Thus, for best adaptation and performance of strains under salinity, isolation and screening of actinobacteria from saline region of IGP was adopted as one of the criteria in the present study.

In the present investigation, accumulation of osmoprotectant/compatible solute proline was recorded in actinobacterial strains which enable them to survive under stressed conditions. In extreme osmotic stress, compatible solutes could be synthesized by microorganisms that act as osmoprotectants. These compatible solutes include soluble sugars, proline and their derivatives, that help the organisms to survive in osmotic stress (Da-Costa et al., 1998; Bacilio et al., 2004; Parida and Das, 2005). The compatible solute proline is synthesized by a wide variety of microbial and plant species as protection against osmotic stress (Wood et al. 2001). In the current study, all the 28 halotolerant isolates were producing osmolyte proline which increased with increasing concentration of salt (NaCl), indicating a probable mechanism (compatible solute accumulation) for stress tolerance. The maximum production of proline was recorded in the isolate K36 followed by isolate K34. Tiwari et al. (2011), also reported accumulation of proline in some bacterial strains *viz. Bacillus pumilus, Pseudomonas mendocina, Arthrobacter sp., Halomonas sp.,* and *Nitrinicola lacisaponensis* isolated from highly saline habitats, for survival in saline stressed conditions. Likewise in a study performed by Upadhyay et al. (2012) it was reported that halotolerant bacterial isolates from saline soil of eastern IGP were adopting osmotolerance mechanism (proline production) in stressed environment to cope up with the adverse conditions and the quantity of proline synthesized was proportional to the NaCl stress, which supports the findings of the present study. The proline content could maintain the growth of bacterial isolates up to higher salinity level because, it may act as a mediator of osmotic adjustment that protects macromolecules during dehydration and serve as a hydroxyl radical scavenger (Csonka 1982; Miller 1996).

Beneficial interactions with bacterial inoculation and differential mechanisms underlying plant growth promotion are documented (Yang et al. 2008; Meena et al. 2010; Egamberdieva et al. 2011). Bacteria isolated from different stressed habitats, possess stress tolerance along with the plant growth-promoting traits and therefore such isolates are potential candidates for stress remediation in plants (Tiwari et al. 2011; Upadhyay et al. 2012; Yandigeri et al. 2012; Ramadoss et al. 2013). In the present study, out of the 28 halotolerant actinobacterial strains, two strains *viz*., K34 (*Streptomyces albogriseolus*) and K36 (*S. griseorubens*) were selected for pot assay with wheat based on their PGP attributes (IAA production, siderophore production, ammonia production) and osmotolerance ability. Both the isolates were found to increase the shoot and root growth in wheat at 15 days after sowing (DAS) under non-saline conditions. The isolates were performing well individually with wheat but the combination of both gave better results with further increase in shoot length and root length. The probable reason might be the synergistic potential of both the isolates to produce IAA, siderophore and ammonia. The results observed are in accordance with the previous reports on beneficial microbes (Nadeem et al. 2009; Tank and Saraf 2010).

A high concentration of salts had a negative impact on seed germination and plant growth by affecting various metabolic processes (Sidari et al. 2008). Salt stress affects all the major plant processes, such as growth, photosynthesis, protein synthesis, energy and lipid metabolism (Parida and Das 2005). Salinity is a major constraint, which hampers wheat production, causing a loss of ~ 65% in yield in moderately saline soils (Shafi et al. 2010). Use of PGPR inoculums for growth of cereals to alleviate salt stress has been reported (Ashraf et al. 2004; Barriuso et al. 2008), but information is scanty on the role of salt-tolerant PGPR in wheat under saline field conditions (Upadhyay and Singh 2015). In the present study, the halotolerant isolates selected for pot assay were mitigating the negative effect of salinity at 15 and 30 DAS. The shoot and root length was found to increase significantly when the isolates were used in combination. The reason might be PGP activities along with the high osmotolerance potential of the isolates. Many researchers have also shown that PGPR strains do improve crop growth under salinity stress conditions (Nadeem et al. 2009; Tank and Saraf 2010).

Proline acts as a potent compatible solute and proline concentration has a direct correlation with salinity. The inoculation has greatly increased the concentration of proline in wheat leaves after exposure to salt stress. The accumulation of proline in response to salt stress has a role in osmotic adjustment and can decrease the water potential to help maintain the water content in leaves. Although in high external salt concentrations this occurs at the expense of plant growth, it may allow the plant to survive salt stress or even to recover (Munns and Tester 2008; Krasensky and Jonak 2012). Accumulation of proline may lead to higher leaf water potential as well as it protects the plants from oxidative stress imposed by salt (Zarea et al. 2012) and proline accumulation maintains redox balance in stress conditions (Ashraf and Foolad 2007).

The yield parameters were also reported to be better in the treatment T3, in which the isolates were used in combination. This is due to the synergistic effect of both the isolates in combination as well as the role of proline so synthesized by the actinobacterial isolates as compatible solute for plants, thus relieving stress in them so as to increase the yield components as compared to control in saline soil.

The data reported in this study are quite encouraging in terms of promoting the use of halotolerant PGP actinobacteria as a means for improving plant growth under field conditions and maximizing the utilization of salt-affected soils for increasing agricultural production from these soils.

Chapter 6

CONCLUSION

Chapter 6

Conclusion

In conclusion, the successive efforts to isolate actinomycetes from saline soils of IGP had been accomplished successfully with twenty eight halotolerant actinobacteria from five districts (Auraiya, Etawah, Mainpuri, Fatehpur, and Kanpur) of Indo-gangetic plains of Uttar Pradesh, India. The selective isolation media and enrichment techniques greatly reduced the unwanted microflora and increased the number of actinobacterial colonies. Knowledge generated on diversity of halotolerant actinobacteria from salt affected soil of IGP, India, revealed the inhabitance of diverse halotolerant actinobacteria in saline soils with predominance of *Streptomyces* genera. This study contributes to the acquaintance of saline soil associated actinobacteria and further augments the array of PGP actinobacterial isolates available for sustainable agricultural practices. As noted in this study, a halotolerant isolate from rare genera of actinomycete was also isolated thus, opening avenues for further isolation of rare actinobacteria from stressed habitat of IGP, which could be utilized as potential sources of secondary metabolites (antibiotics). The present investigation revealed the multifunctional properties of halotolerant actinomycetes. 75% of them produced the enzyme amylase and 57.14% produced protease enzyme. In total, 28.57%, 35.71% and 85.71% produced gelatinase, cellulase and urease, respectively. Hydrogen sulfide (H_2S) production was recorded in 39.28% of the isolates. Eleven out of total twenty eight isolates were capable of producing hydrogen sulfide as observed from a positive isolate by black colouration on the SIM agar media. A total of 42.85% isolates produced siderophore, the iron chelating molecule. 78.57% were ammonia producers while only 21.42% of the isolates were producing HCN. Although phosphate solubilization and chitinase production was detected in 28.57% and 7.14% of the isolates respectively but interestingly, Indole acetic acid (IAA) production was detected in all the 28 halotolerant isolates and this enhanced in the presence of salt upto 6% w/v. Only two isolates (K17 and K20) were found to have chitinase activity as seen by a clearing zone around the streaked colony, which was further confirmed by the presence of *chiA* gene. Of all the 28 isolates tested for their biocontrol activity against plant pathogen *Sclerotium rolfsii* and *Macrophomina phaseolina*, the two isolates K17 and K20 which possessed chitinase activity were found to effectively control the two pathogens. Scanning Electron Microscopy further confirmed the finding. Based on 16S rRNA partial gene sequencing of the 28 halotolerant, they were grouped into two genera belonging to two families, *viz*., Streptomycetaceae and Pseudonocardiaceae. Of this, 26 isolates belonged to the genera *Streptomyces* while one belonged to the genera *Actinoalloteichous* and remaining one was identified as *Actinomycete*. Based on the ARDRA pattern, all the isolates were grouped into eight phenons (3 major and 5 minor). All the twenty eight sequences have been allotted accession number by NCBI GenBank and are in public domain. Osmotolerant proline content enhanced in presence of salt with strains K34 and K36 being the best producers under normal as well as stressed conditions. Further in this study both halotolerant *Streptomyces* (K34 and K36) isolates in consortium gave promising results for growth and yield in wheat under saline condition, therefore, can be utilized in the field as biofertilizers for sustainable agricultural practices and management of the saline soil. The results of the current study may serve as baseline data for selecting the actinobacterial strains for management of saline soil and fungal pathogens thus, magnetising the researches in the field of biofertilizers and biocontrol agents.

Chapter 7

SUMMARY

Chapter 7

Summary

Soil salinity is a worldwide environmental problem that adversely affects crop productivity as well as quality of produce in arid, semi-arid regions and in coastal areas (Rengasamy 2010). Approximately 20% of the irrigated crop area in the world has been reported to be affected by salinity, and the situation is continuously deteriorating (Hasanuzzaman et al. 2013). According to the FAO Land and Nutrition Management Service (2008), over 6% of the global areas are affected by either salinity or sodicity accounting for more than 800 million ha of land (Yadav et al. 2011). Between the years 2015 and 2050 the global population is expected to increase to ~10 billion. This increase in population, along with rising economics, will exert greater pressure on demands for crop and livestock products. Currently, the growth rates in the yields of maize, rice, wheat, and soybean are insufficient to meet projected food demands by 2050 (Ray et al., 2013). The causes of declining growth rates include increasing pressure from salinity and water logging, depletion of soil nutrients and organic matter, climate change, and inappropriate crop management practices (Wichelns and Qadir, 2015).

The Indo-gangetic plain (IGP) is one of the most extensive fluvial plains of the world. It accounts for the one third cultivable lands and is a major contributor of the food supply for the Indian population (Shrivastava et al. 2015). The Indo-Gangetic plain is characterized by intensive agriculture, largely by resource-poor small and marginal farmers. By the year 2050, the Indo-Gangetic region, India's bread basket, will face intense pressure from uncontrolled population growth, in terms of land and water resources, both of which are vital elements for sustainable agricultural growth (FAO, 2011). During the past five decades, various soil-forming processes such as calcification, leaching, lessivage, salinization and alkalinization, gleization and homogenization have been identified in the IGP (Srivastava et al. 2015). Vast swatches of salt-affected areas in the region provide both challenges and opportunities to bolster food security. Due to the emergence of environmental pollution and health concerns for both producers and consumers by exhaustive use of chemicals for saline soil reclamation, developing salt-tolerant crops has been a much desired scientific goal in recent times. However it has met with little success to date, as only few major-determinant genetic traits of salt tolerance have been identified (Munns and Tester 2008; Schubert et al. 2009). An alternative strategy to improve crop salt tolerance may be to introduce salt-tolerant microbes that could provide an effective eco-friendly approach to reduce environmental and health problems and enhance crop growth. Since soil microbes have been reported to enhance the growth of many different crops grown in a wide range of root-zone salinities, this approach may succeed where it has proved difficult to develop salt-tolerant lines. Indeed, several recent studies have demonstrated that local adaptation of plants to their environment is driven by genetic differentiation in closely associated microbes (Rodriguez and Redman 2008).

Actinomycetes (actinobacteria) are Gram positive bacteria that have a great capacity to survive in adverse environments such as saline soils and are reported to possess plant growth promoting properties. Among them, halophilic and halotolerant actinobacteria are recently gaining much attentions. Despite this, only few reports are available on actinomycetes addressing the issue of plant growth promotion in saline soil. Metabolites and biological functions from halophilic or halotolerant members of this bacterial group may resolve the ever increasing thirst of industry for metabolites with salt tolerant capacity to combat a range of issues from environmental pollution to disease and hunger (Hamedi et al. 2015). The present study was undertaken with the objectives to isolate and characterize halotolerant actinomycetes from stressed habitats with plant growth promotion potential, proline production capability and their evaluation under pot assay for growth promotion of wheat plants in saline stressed condition.

Saline soil samples were collected from salt affected regions of five districts of IGP including Fatehpur, Kanpur, Auraiya, Etawah and Mainpuri, Uttar Pradesh, India. Pre-treatment of soil samples was done for isolation of actinobacteria and suppression of other unwanted microbes. A total of 55 isolated actinobacteria were screened for their *in vitro* salt tolerance capacity by evaluating their growth on NaCl amended media. 28 halotolerant/halophilic isolates were selected for further study.

The halotolerant strains were evaluated for their plant growth promotion traits *viz.* indole acetic acid (IAA) production, P-solubilisation, siderophore, HCN, chitinase and ammonia production. Biocontrol potential of isolates was tested against two fungal phytopathogens (*M. phaseolina* and *Sclerotium rolfsii*) by dual culture assay as well as interaction study using scanning electron microscopy. The morphological characterisation and biochemical tests (such as amylase, protease, cellulase, urease, gelatinase and H_2S production) were performed for the preliminary identification of the isolates. The special characteristics of the halotolerant isolates were determined by study of their growth at different pH and temperature and physiological characterisation was done by studying carbon utilization profile. The halotolerant isolates were characterised using molecular tool *i.e.* 16S rDNA amplification followed by Restriction Fragment Length Polymorphism (RFLP). Finally, the identity of the isolates was confirmed by 16S rDNA sequencing and the nucleotide sequences of 16S rRNA gene were deposited in NCBI GenBank. PCR amplification for hydrolytic enzyme chitinase (*chiA*) gene was performed using the gene specific PCR primers. For *in vivo* pot assay two isolates K34 and K36 were selected based on their PGP attributes and proline production capacity under stressed conditions. The isolates were analysed for their plant growth promotion ability with wheat in non-saline and saline conditions (soil pH- 9.2; EC- 4.62 dS/m). Wheat seeds were procured from Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. The pot assay was performed in natural environmental conditions.

The salient results of this study are summarised below:

Out of 55 actinobacteria isolated from salt affected soil samples, 28 isolates were halotolerant with optimum growth at 8% (w/v), NaCl concentration. Since these 28 isolates exhibited good growth in absence or presence of salt, such isolates were designated as moderately (intermediate) halotolerant. All 28 halotolerant isolates were recorded as Indole Acetic Acid (IAA) producers and in two isolates *viz.* K34 and K36 the quantity of IAA produced was directly proportional to salt concentration up to 6% (w/v) NaCl level. A decline was recorded in IAA production beyond 6% NaCl. A total of 22 isolates recorded as ammonia producer, 12 isolates showed siderophore production, eight isolates phosphate solubilisation, six isolates recorded HCN production and chitinase activity was reported only in two isolates. Biocontrol assay against the selected phytopathogen revealed that only two isolates possess antifungal activity; the isolate K20 against *M. phaseolina* and K17 against *Sclerotium rolfsii*. Both the isolates exhibited chitinase activity which was further substantiated by the amplification of 270bp product specific for *chiA* gene in the isolates. The microscopic (SEM) examination of the antagonistic interactions of strains K20 and K17 revealed these strains were entering the fungal mycelia causing distortions and injuries leading to destruction of the mycelia.

The results of phenotypic characterisation of all the 28 halotolerant actinobacterial isolates showed that all of them had hard embedded colonies and varied in the color of aerial and substrate mycelia; 17 isolates having aerial mycelia with grey spores, four isolates having pink spores, three had creamy aerial spore mass, two with white aerial spore mass, and one each had ivory and ash brown aerial spore mass. Soluble pigment production was reported in five isolates. Spore ornamentation studies revealed that most of the isolates had smooth spores. However four isolates, *viz*., K4, K5, K9 and K24 had spiny spores while one isolate K30 had hairy spore morphology. Four different types of spore chains such as straight, rectiflexibilis, retinaculiperti and spirals were observed in the isolates. All 28 isolates were able to grow at a temperature range of 25-50^{o}C and pH range of 5.0-11.0 revealing their moderately alkali-tolerant nature. Out of 10 different sugars tested for carbon utilization, all the isolates were capable of utilizing a minimum of six different sugars as a carbon source. All the 28 halotolerant isolates were producing at least one of the industrially important enzymes *viz.* amylase, protease, cellulase and gelatinase and based on biochemical observations, these strains could be assigned to the *Streptomyces* genera.

Amplification of 16S rDNA was carried out and a single amplicon of about 1150 bp was found in all the isolates. PCR amplification yielded good amount of PCR products which was sufficient for digestion with different tetracutters like *Taq*I, *Hae*III and *Msp*I. The characterization of these isolates on the basis of ARDRA produced polymorphic patterns with different profiles characterized by 3-4 fragments ranging from 50 bp to 450 bp for different isolates. The enzymes, *Hae*III and *Msp*I were found to be more discriminative compared to *Taq*I. Based on 16S rRNA gene partial sequencing, similarity values of >98% suggested that 26 strains belonged to genus *Streptomyces*, and one each strain to *Actinomycete* and to the rare genera *Actinoalloteichus*.

Quantitative estimation of osmolyte (Proline) synthesis in all the 28 halotolerant isolates revealed that all were producing proline to combat adverse effects of salinity stress and the amount of proline synthesized was increasing with higher salinity with maxima in isolate K36 followed by K34. Based on PGP potential and proline synthesis in stressed condition, *in vivo* study done in wheat with *Streptomyces* isolates K34 and K36 revealed that both the isolates were enhancing the growth parameters in wheat under non-saline and saline conditions, individually however, better results were reported when both the isolates were used together. Combination of both the isolates even increased the grain yield of wheat in saline conditions by 109.4% as compared to control.

The present study was successful in isolating and selecting halotolerant actinobacterial strains from salt-affected regions of IGP, Uttar Pradesh, India having plant growth promotion potential and other important attributes. The results not only identified the isolates with PGP potential but also detected strong correlation between salinity and IAA and proline production in isolates. The diversity and functional potential of actinobacterial strains with phosphate mineralisation ability, plant growth promotion potential and biocontrol properties was obtained in the study. Knowledge generated on biodiversity of *Streptomyces* strains with different PGP potential will be helpful to design strategies to use these strains as bio-inoculants for effective management of salinity affected lands. On the basis of above results, the two halotolerant *Streptomyces* strains could be exploited as consortium for sustainable agriculture practice for bioremediation of saline-sodic soil.

Concisely, present study offers the overview on salt tolerance, plant growth promotory activity and molecular phylogeny of halotolerant actinobacteria isolated from the salt affected soil of IGP, Uttar Pradesh, India. The successive efforts to study the diversity of halotolerant actinobacteria from salt affected soil of IGP, U.P. India, revealed the inhabitance of diverse actinobacteria in saline soil with predominance of *Streptomyces* genera. This study contributes to our acquaintance of saline soil associated actinobacteria and further augments the array of PGP actinobacterial isolates available for sustainable agricultural practices for alleviating deleterious effects of salinity on crops (with special reference to wheat) and our observations certify the beneficial role of *Streptomyces* isolates for the plant growth promotion under salinity stress. Moreover, there is much that will still need to be done, but the findings of this work will form a strong foundation and may serve as a baseline data for enhancing the growth of plants in salinity compromised soil, for bio-management of such soils using halotolerant plant growth promoting actinobacteria.

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APPENDIX

APPENDIX-I

Starch Casein Agar

Starch Soluble	10.0 g
K_2HPO_4	2.0 g
KNO_3	2.0 g
NaCl	2.0 g
Casein	0.3 g
$MgSO_4.7H_2O$	0.05 g
$CaCO_3$	0.02 g
$FeSO_4$.7H2O	0.01 g
Agar	15.0 g
Distilled water	1000 mL
pH	7.5±0.2

Actinomycetes Isolation Agar

Sodium caseinate	2.0 g
Asparagine	0.1 g
Sodium Propionate	4.0 g
Dipotassium phosphate	0.5 g
$MgSO_4.7H_2O$	0.1 g
$FeSO_4$	0.001 g
Agar	15.0 g
Glycerol	5.0 g
Distilled water	1000 mL
pH	7.5±0.2

Humic Acid Vitamin Agar

Humic acid	1.0 g
Na_2HPO_4	0.5 g
KCl	1.71g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4$.7H2O	0.01 g
$CaCO_3$	0.02 g
Agar	18.0 g
Distilled water	1000 mL
pH	7.2±0.2

Add B-Vitamins after mixing all components

Thiamine hydrochloride	0.5 µg
Riboflavin	0.5 µg
Niacin	0.5 µg
Pyridoxine hydrochloride	0.5 µg
Inositol	0.5 µg
Calcium pantothenate	0.5 µg
Para amino benzoic acid	0.5 µg
Biotin	0.25 µg

Carbohydrate Utilization Basal Media

$(NH_4)_2 SO_4$	2.64 g
KH_2PO_4.(anhydrous)	2.38 g
$K_2HPO_4.3H_2O$	5.56 g
$MgSO_4.7H_2O$	1.00 g
$CuSO_4.5H_2O$	0.0064 g
$FeSO_4.7H_2O$	0.0011 g
$MnCl_2.4H_2O$	0.0079 g
$ZnSO_4.7H_2O$	0.0015 g
Agar	18.0 g
Distilled water	1000 mL
pH	6.8-7.0

Reese’s mineral media with starch

KH2PO4	2.0 g
$(NH_4)_2SO_4$	1.4 g
Starch	10.0 g
$MgSO_4.7H_2O$	0.3 g
$CaCl_2$	0.3 g
$FeSO_4.7H_2O$	5.0 g
$MnSO_4.H_2O$	1.6 mg
$CoCl_2$	2.0 mg
$ZnSO_4.7H_2O$	1.4 mg
Distilled water	1000 mL
Agar	18 g
pH	7.0

Gelatin Medium :

Beef Extract	3.0 g
Peptone	5.0 g
Gelatin	12.0 g
Agar	10.0 g
Distilled water	1000 mL
pH	7.0

Urea Agar

Glucose	1.0 g
Casein-Peptone	1.0 g
$Na_2HPO_4.2H_2O$	1.9 g
KH_2PO_2	1.5 g
$MgSO_4.7H_20$	0.5 g
NaCl	5.0 g
Phenol red	0.012 g
Agar	12.0 g
Distilled water	900 mL
pH	7.0

Solution of urea

Urea	10.0 g
Sterile water	100 mL

SIM Agar

Peptone	3.0 g
Beef extract	3.0 g
$(NH_4)_2.SO_4$	0.2 g
$FeSO_4.$	0.25 g
Agar	5.0 g
Distilled water	1000 mL
pH	7.3±0.2

Skim Milk Agar

Pancreatic digest of casein	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Skim Milk	25.0 g
Agar	15.0 g
Distilled water	1000 mL
pH	7.0

Reese’s mineral media with carboxymethylcellulose

$FeSO_4.7H_2O$	0.5 g
$FeSO_4$	0.01 g
$NaNo_3$	2.0 g
CMC	10.0 g
K_2HPO_4.	0.05 g
$CaCl_2$	0. 02 g
$MnSO_4$	0.02 g
Agar	18.0 g
Distilled water	1000 mL
pH	7.0

Glucose Yeast Extract Malt Extract Broth

Malt Extract	10.0 g
Yeast Extract	4.0 g
Glucose	4.0 g
Distilled water	1000 mL
pH	7.3±0.2

Pikovskaya’s Medium

Glucose	10 g
Yeast extract	0.05 g
$(NH_4)_2SO_4$	0.5 g
Kcl	0.2 g
NaCl	0.2 g
$MgSO_4.7H_20$	0.1 g
$FeSO_4.7H_2O$	Trace
$MnSO_4 7H_2O$	Trace
$Ca_3(PO_4)_2$	5.0 g
Agar	18.0 g
Distilled water	1000 mL
pH	7.0±0.2

Peptone water broth

Peptone	10.0 g
NaCl	5.0 g
Distilled water	1000 mL
pH	7.0±0.2

Nutrient Broth

Peptone	5.0 g
Beef extract	3.0 g
NaCl	3.0 g
Distilled water	1000 mL
pH	7.4±0.2

APPENDIX-II

A) Reagents for Molecular studies

Lysis Buffer

Tris HCl	50 mM
EDTA	50 mM
SDS	3%
2-mercapto ethanol	1%
pH	7.3

TE Buffer

Tris HCl	10 mM
EDTA	1 mM
pH	8.0

TAE (50X)

Tris base	242 g
Glacial acetic acid	57.1 mL
0.5M EDTA (pH 8.0)	100 mL
Distilled H2O	1000 mL

Gel loading Buffer (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	30 mL
Distilled Water	to 70 mL

2X Gel Loading Dye

Bromophenol blue	0.05 g
Xylene cyanol	0.05 g
Formamide	9.5 mL
0.5 M EDTA	0.4 mL
pH	8.0
Total volume	10.0 mL

B) DNA Isolation by Lysis Buffer Protocol

- The cells were grown in 50 mL of nutrient broth for 48 h at 37 °C in an orbital shaker.
- Cells were harvested by centrifugation (5 min. 10, 000 rpm) and cell pellet is rinsed with TE buffer, this process was repeated two or three times.
- Pellet was resuspended in 500 µL SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5) in eppendorf tubes.
- Added 15 µL of lysozyme (10 mg/ mL conc.) to above and incubated at 37°C for 2-3 h.
- 1/10th (0.1 volume) volume of 10% Sodium Dodecyl Sulphate (SDS) and 5 µL of RNase A (10 mg/mL) was added and incubated at 37 °C for 15 min.
- 15 µL of Proteinase K (10 mg/ mL) was added and incubate at 55 °C for 3-4 h or overnight.
- 1/3 (or 0.3) volume of 5 M NaCl is added, 1 volume of water saturated phenol and chloroform: isoamyl alcohol (24:1) is used to incubate at room temperature for 30 min with frequent inversion.
- Centrifugation was done at 12,000 rpm for 15 min and the aqueous layer was transferred to a new tube using a blunt-ended pipette tip to reduce shearing of the DNA.
- Add 0.1 volume of 3M Sodium acetate (CH3COONa) (pH- 4.8) and 1 volume of chilled absolute ethanol.
- DNA was precipitated gently inverted the tube and let stand at room temperature for 30 min.
- DNA was pelleted by centrifugation at 12,000 rpm for 5 minutes at 4 °C.
- Pellet was washed with 70% ethanol at 12,000 rpm for 5 minutes at 4°C.
- Washing step was done twice to eliminate the salts.
- Pellet in the tubes was air dried for 4 hrs to complete removal of ethanol and 40 µL TE buffer or sterile water (Protease, Nuclease free) (GeNei). was added.
- The DNA samples were stored at -20ºC till further use.

- 2% sodium carbonate in 0.1N sodium Hydroxide (Reagent A)
- 0.5% copper sulphate (CuSO4.5H2O) in 1% potassium sodium tartrate (Reagent B)
- Alkaline copper solution: Mix 50 mL of A and 1 mL of B prior to use (Reagent C)
- Folin- Ciocalteau (Reagent D)

APPENDIX-IV

S.No.	**16S rRNA partial Sequences**
1.	>K2 (*Streptomyces fradiae*) KC511795 CGGCCCTCCCTTATAGTTTGGTCCTGGCTCCGAGGGGCCCACTTCCCGTGGGTAGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACCACTTCAGGCATCTGATGGTGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
2.	>K3 (*Streptomyces fradiae*) KP331747 GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGCAAGGTGGAGCGAATTCAAAAAGCCGGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCC
3.	>K4 (*Streptomyces coeruleorubidus*) KP331748 CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC
4.	>K5 (*Streptomyces thermolilacinus*) KP331749 ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACACCCAGAGATGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGTCTGCAACTCGACCCCATGAAGT
5.	>K8 (*Streptomyces atrovirens*) KP331750 ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAG

CTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAG
CTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCG
GAAACGTCCAGAGATGGGCGCCCCCTTGTGGTCGGTGTACGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCCGCAACGAGCGCAAACCCTTGTCCCGTGTTGCCAGCAAAGCCCTTTCGGGGT
GTTGGGGACTCACGGGAGACCGCCGGGG

6. >K9 (*Streptomyces gancidicus*) KP331751
GGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACTGCAGAAGAA
GCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGT
AAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATAC
GGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTG
CCGCACTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATAC
ACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTG

7. >K10 (*Streptomyces thermoluteus*) KP331752
ACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTG
AAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAAT
TCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACGGTGGCGAAGGCGGATCTCTGGGCCGATA
CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTG
GGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTC
GACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGCCCCCTTGTG
GTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGTCCCGTGTTGCC

8. >K12 (*Streptomyces celluloflavus*) KC511796
ATGAAACCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCTTCACTCTGGG
ACAAGCCCTGGAAACGGGGTCTAATACCGGATAATACTCCTGCCTGCATGGGTGGGGGTTGAAAGCTCCGGC
GGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTATGGCCTACCAAGGCGACGACGGGTAGCCG
GCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
CTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCC
GCGGAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTC
GGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGAT
CGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGGTGGCGAAGGCGGATCTCTG
GGGCCATTACTGACGCTGACGACCCAAAGC

9. >K13 (*Streptomyces mutabilis*) KP331754
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCGCGTGAGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCG
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAA
GAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGG
CAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC
ACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
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CAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACAC
CGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCT
GGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTT
ATGTCTTGGGCTGACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCA
AAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTC

10. >K14 (*Streptomyces althioticus*) KP331755
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCGCGTGAGGG
ATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGC
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AGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGG
GCAGGCTAGAGTTCGGTAGGGGAGCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACA
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CCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACC
GGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTG
GGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTA
TGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCA
AAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAG

11. >K16 (*Streptomyces anandii*) KP331756
TTTCAGAGGCCTCCCCTCTTGGCCGGTTCCCAGGCGGACTTCCCGGTACCTGCAGAAGAAGCGCCGGCTAACT

ACGTGCCAGCAGCCGCGGCAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGAAAGAGCTCGTAG
GCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAG
TTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCG
AAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAAATACCCT
GGTAGTCCGCGCCGTAAACGGAGGGCACTAGGTGTGGGCCACATTCCACGTCGTCCGTGCCGCAGCTAACGC
ATTAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAGCG
GCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTT

12. >K17 (*Streptomyces rochei*) KP331757
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGT
ACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCG
GAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGG
TCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGC
AGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATT
CCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCCAGGCTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTAC
CAAGGCTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCCGCAACGAGCGCAACCCTTGTCCCCGTGTTGCC
AGCAGGCCCTTGTGGGTGCTGGGGACTCACGGAAGACCGCCGGGGTCCAACTCGGAGGAAGGTGGGGGACG
ACGTCAAGTCATCATGCCCCCTTTATGTCTTGGG

13. >K20 (*Streptomyces aureofaciens*) KP331758
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGC
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAG
AGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGC
AGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACA
CCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGC
AGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACC
GGAAAGCATCAGAGATGGGGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCCTT

14. >K21 (*Streptomyces carpaticus*) KC511797
CGGGCGTCCTTAAGACATGCAGTCGAACGATGAACCGGTTTCGCCGGTGATTAGTGGCGAACGGGTGAGTAA
CACGTGGGCAATCTGCCTGCACTCTGGGATAAGCCCGGGAAACTGGGTCTAATACCGGATACGACACTCCGA
GGCATCTTGGGGTGTGGAAAGTTCCGGCGGTGCAGGATGAGCCCGCGCCTATCAGCTTGTTGGTGGGGTAAT
GGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTG
AGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAG
AAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGC
GTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGATTGTGAAAGCCCGGGGCTTAACCCTGGGTCTGCAGTCGAT
ACGGGCAGGCTAGAGTTCGGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGA
GGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCG
TGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGAC
ATACATCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCAACCTCTTCG
GAGGGTTGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCAT
GCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGC
GAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCC

15. >K22 (*Streptomyces rubrolavendulae*) KC511798
GTCGCGTCTTGAGACATGCAGTCGTCCGATGAACCCGCTTCGCTGGGGGATTAGTGGCGAACGGGTGAGTAA
CACGTGGGCAATCTGCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACCACTTCA
GGCATCTGATGGTGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTA
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CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGTGATGCAGCGACGCCGCGTG
AGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAG
AAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGGTTGTCCGGAATTATTGGGCG
TAAAGAGCTCGTAGGCGGCCTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGTCTGCATTCGATAC
GGGCAGGCTAGCGTTCGGAAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTCGTCCGTG
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GGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACAT
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GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGG
TGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCC
CCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGCAAGGTGGAGCGAA
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GCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGG
TAACACCC

16. > K24 (*Streptomyces viridosporus*) KP331760
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
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AAACCCCCGGAGACGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCCTTCGGGGGTGC
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CAAAAAGCCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCCATGAAGTCGGAGTCGCTAGTAAA

17. >K26 (*Streptomyces albogriseolus*) KP331761
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGG
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AGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGG
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TTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCC
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CCGGAAACGTCCAGAGATGGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGT
GTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCC
CCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAA
TCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCC

18. >K28 (*Streptomyces coelicolor*) KP331762
GTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGGCCTAATACGTAGGGCGCGAGCGTTGTCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGG
GTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCG
CAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATT
CCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTAC
CAAGGCTTGACATACACCGGAAA

19. >K29 (Actinomycete) KP331763
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
GGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGC
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AAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGG
GGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTAT
GTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAA
AAAAGCCGGT

20. >K30 (*Streptomyces acrimycini*) KP331764
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA
CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAG
CTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAG
CTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC
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GAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGTGTTG
GGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTA
TGTCTTGGGCTGCACACGTGCTACAATGGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTC
AAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCCACTCCGACCCCCATGA

21. >K31 (*Streptomyces rameus*) KP331765
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGG
ATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGC
GCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAA

AGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGG
GCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAA
CACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCC
GCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACA
CCGGAAACGTCCAGAGATGGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTG
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TGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCT
TATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGCGAGGTGGAGCGAATCT
CAAAAAAGCCCGGTCTCAGTTCGGATTGGGGTCCTGCAACTCGACCCCCAT

22. >K32 (*Streptomyces coelicoflavus*) KP331766
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
GGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGC
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TACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGC
TAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGG
AAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTGTCCCGTGTTGCCAGCAACTCTTCGGAGGTTGG
GGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTAT
GTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAA
AAAGCCGGTCTCAGTTCGGATTGGGGTCTGCACTCGACCCCCATGAAGTCGGAGTCGCTA

23. >K33 (*Streptomyces fradiae*) KP331767
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
GGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGC
TCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGG
CTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
GTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGC
TAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGG
AAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAACTCTTCGGAGGTTGGG
GACTCACGGGAGACCGCCGGGGTCAACTCGTGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATG
TCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAA
AAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTA

24. >K34 (*Streptomyces albogriseolus*) KC511799
TTCACCCCAATCCATAAAGAAACACAAAGTCTTTTATTTCGGACATTATTGTCTGGCTAGGGGGCTGGTTCAG
TGGGTTATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAA
CGGGGTCTAATACCGGATACTGACCCGCTTGGGCATCCAAGCGGTTCGAAAGCTCCGGCGGTGCAGGATGAG
CCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGC
GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTGCGGGAGGCAGCAGTGGGGGATATTGCACAAT
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CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGC
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GCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGCCCCCTTGTGGTCG
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CCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGA
GGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTA
CAATGAGCTGCGA

25. >K35 (*Streptomyces coeruleorubidus*) KP331769
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAG
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CGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATAC
ACCGGAAACGTCTAGAGATGGGGGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTG
CTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCC

TTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATC
TCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTA

26. >K36 (*Streptomyces griseorubens*) KC511800
GGGGCAGCGTTATAGTTTGCAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAAC
ACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGACCCGCTT
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ATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGC
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GAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCAAAGCTGGGGGGGAAAA
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27. >K37 (*Streptomyces diastaticus*) KP331771
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
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TCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGG
CTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
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CTTGGGCTGCACACGTGCTACTTTGGCCGGTACCCTGAGCTGCGATGATGCAAGGTGGAGCGAAT

28. >K38 (*Actinoalloteichus cyanogriseus*) KP331772
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCGCCGAAGAAGCGAAAGTGACGGTAGGCGCAGAAGAA
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GGGCAGACTTGAGTTCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGGGATTTCCACGTCCTCCGTGC
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ACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGAGGTCAAGTCATCATGCCCCTTATGTC
CAGGGCTTCACACATGCTACAATGGCCGGGTACAGAGGGCTGCGAAATCGCAAGGTGGAGCGAATCTCTTAA
GGCCGGTCTCAGTTCGGATCGGGGTTCTGCAACTCGACCCCGTGAAG

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REVIEW

Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation

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Salt-tolerant;
PGPR;
Microorganisms

Abstract Salinity is one of the most brutal environmental factors limiting the productivity of crop plants because most of the crop plants are sensitive to salinity caused by high concentrations of salts in the soil, and the area of land affected by it is increasing day by day. For all important crops, average yields are only a fraction – somewhere between 20% and 50% of record yields; these losses are mostly due to drought and high soil salinity, environmental conditions which will worsen in many regions because of global climate change. A wide range of adaptations and mitigation strategies are required to cope with such impacts. Efficient resource management and crop/livestock improvement for evolving better breeds can help to overcome salinity stress. However, such strategies being long drawn and cost intensive, there is a need to develop simple and low cost biological methods for salinity stress management, which can be used on short term basis. Microorganisms could play a significant role in this respect, if we exploit their unique properties such as tolerance to saline conditions, genetic diversity, synthesis of compatible solutes, production of plant growth promoting hormones, bio-control potential, and their interaction with crop plants.

Contents

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1. Introduction

The beginning of 21st century is marked by global scarcity of water resources, environmental pollution and increased salinization of soil and water. Increasing human population and reduction in land available for cultivation are two threats for agricultural sustainability (Shahbaz and Ashraf, 2013). Various environmental stresses *viz*. high winds, extreme temperatures, soil salinity, drought and flood have affected the production and cultivation of agricultural crops, among these soil salinity is one of the most devastating environmental stresses, which causes major reductions in cultivated land area, crop productivity and quality (Yamaguchi and Blumwald, 2005; Shahbaz and Ashraf, 2013). A saline soil is generally defined as one in which the electrical conductivity (EC) of the saturation extract (EC_e) in the root zone exceeds 4 dS m^{-1} (approximately 40 mM NaCl) at 25 °C and has an exchangeable sodium of 15%. The yield of most crop plants is reduced at this EC_e, though many crops exhibit yield reduction at lower EC_es (Munns, 2005; Jamil et al., 2011). It has been estimated that worldwide 20% of total cultivated and 33% of irrigated agricultural lands are afflicted by high salinity. Furthermore, the salinized areas are increasing at a rate of 10% annually for various reasons, including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices. It has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Jamil et al., 2011).

Water and soil management practices have facilitated agricultural production on soil marginalized by salinity but an additional gain from these approaches seems problematic (Zahir et al., 2008). Impacted soils are a major limiting production factor worldwide for every major crop (Bacilio et al., 2004; Shannon and Grieve, 1999). A significant increase (an estimated 50%) in grain yields of major crop plants such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) is required to fulfill the food supply requirements for the projected population by 2050 (Godfray et al., 2010). The urgency of feeding the world's growing population while combating soil pollution, salinization, and desertification has given plant and soil productivity research vital importance. Under such circumstances, it requires suitable biotechnology not only to improve crop productivity but also to improve soil health through interactions of plant roots and soil microorganisms (Lugtenberg et al., 2002).

Salt stressed soils are known to suppress the growth of plants (Paul, 2012). Plants in their natural environment are colonized both by endocellular and intracellular microorganisms (Gray and Smith, 2005). Rhizosphere microorganisms, particularly beneficial bacteria and fungi, can improve plant performance under stress environments and, consequently, enhance yield both directly and indirectly (Dimkpa et al., 2009). Some plant growth-promoting rhizobacteria (PGPR) may exert a direct stimulation on plant growth and development by providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate (Hayat et al., 2010). Others do this indirectly by protecting the plant against soil-borne diseases, most of which are caused by pathogenic fungi (Lutgtenberg and Kamilova, 2009). The problem of soil salinization is a scourge for agricultural productivity worldwide. Crops grown on saline soils suffer on an account of high osmotic stress, nutritional disorders and toxicities, poor soil physical conditions and reduced crop productivity. The present review focuses on the enhancement of productivity under stressed conditions and increased resistance of plants against salinity stress by application of plant growth promoting microorganisms.

2. Problem of soil salinization

Soil salinity is an enormous problem for agriculture under irrigation. In the hot and dry regions of the world the soils are frequently saline with low agricultural potential. In these areas most crops are grown under irrigation, and to exacerbate the problem, inadequate irrigation management leads to secondary salinization that affects 20% of irrigated land worldwide (Glick et al., 2007). Irrigated agriculture is a major human activity, which often leads to secondary salinization of land and water resources in arid and semi-arid conditions. Salts in the soil occur as ions (electrically charged forms of atoms or compounds). Ions are released from weathering minerals in the soil. They may also be applied through irrigation water or as fertilizers, or sometimes migrate upward in the soil from shallow groundwater. When precipitation is insufficient to leach ions from the soil profile, salts accumulate in the soil resulting soil salinity (Blaylock et al., 1994). All soils contain some water-soluble salts. Plants absorb essential nutrients in the form of soluble salts, but excessive accumulation strongly suppresses the plant growth. During the last century, physical, chemical and/or biological land degradation processes have resulted in serious consequences to global natural resources (e.g. compaction, inorganic/organic contamination, and diminished microbial activity/diversity). The area under the affected soils continues to increase each year due to introduction of irrigation in new areas (Patel et al., 2011).

Salinization is recognized as the main threats to environmental resources and human health in many countries, affecting almost 1 billion ha worldwide/globally representing about 7% of earth's continental extent, approximately 10 times the size of a country like Venezuela or 20 times the size of France (Metternicht and Zinck, 2003; Yensen, 2008). It has been estimated that an approximate area of 7 million hectares of land is covered by saline soil in India (Patel et al., 2011). Most of which occurs in indogangetic plane that covers the states of

Punjab, Haryana, U.P. Bihar and some parts of Rajasthan. Arid tracts of Gujarat and Rajasthan and semi-arid tracts of Gujarat, Madhya Pradesh, Maharashtra, Karnataka and Andhra Pradesh are also largely affected by saline lands.

3. Impact of salinity on plants

Agricultural crops exhibit a spectrum of responses under salt stress. Salinity not only decreases the agricultural production of most crops, but also, effects soil physicochemical properties, and ecological balance of the area. The impacts of salinity include—low agricultural productivity, low economic returns and soil erosions, (Hu and Schmidhalter, 2002). Salinity effects are the results of complex interactions among morphological, physiological, and biochemical processes including seed germination, plant growth, and water and nutrient uptake (Akbarimoghaddam et al., 2011; Singh and Chatrath, 2001). Salinity affects almost all aspects of plant development including: germination, vegetative growth and reproductive development. Soil salinity imposes ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants, and thus limits water uptake from soil. Soil salinity significantly reduces plant phosphorus (P) uptake because phosphate ions precipitate with Ca ions (Bano and Fatima, 2009). Some elements, such as sodium, chlorine, and boron, have specific toxic effects on plants. Excessive accumulation of sodium in cell walls can rapidly lead to osmotic stress and cell death (Munns, 2002). Plants sensitive to these elements may be affected at relatively low salt concentrations if the soil contains enough of the toxic element. Because many salts are also plant nutrients, high salt levels in the soil can upset the nutrient balance in the plant or interfere with the uptake of some nutrients (Blaylock et al., 1994). Salinity also affects photosynthesis mainly through a reduction in leaf area, chlorophyll content and stomatal conductance, and to a lesser extent through a decrease in photosystem II efficiency (Netondo et al., 2004). Salinity adversely affects reproductive development by inhabiting microsporogenesis and stamen filament elongation, enhancing programed cell death in some tissue types, ovule abortion and senescence of fertilized embryos. The saline growth medium causes many adverse effects on plant growth, due to a low osmotic potential of soil solution (osmotic stress), specific ion effects (salt stress), nutritional imbalances, or a combination of these factors (Ashraf, 2004). All these factors cause adverse effects on plant growth and development at physiological and biochemical levels (Munns and James, 2003), and at the molecular level (Tester and Davenport, 2003).

In order to assess the tolerance of plants to salinity stress, growth or survival of the plant is measured because it integrates the up- or down-regulation of many physiological mechanisms occurring within the plant. Osmotic balance is essential for plants growing in saline medium. Failure of this balance results in loss of turgidity, cell dehydration and ultimately, death of cells. On the other hand, adverse effects of salinity on plant growth may also result from impairment of the supply of photosynthetic assimilates or hormones to the growing tissues (Ashraf, 2004). Ion toxicity is the result of replacement of K^+ by Na^+ in biochemical reactions, and Na^+ and Cl^- induced conformational changes in proteins. For several enzymes, K^+ acts as cofactor and cannot be substituted by Na^+. High K^+ concentration is also required for binding tRNA to ribosomes and thus protein synthesis (Zhu, 2002). Ion toxicity and osmotic stress cause metabolic imbalance, which in turn leads to oxidative stress (Chinnusamy et al., 2006). The adverse effects of salinity on plant development are more profound during the reproductive phase. Wheat plants stressed at 100–175 mM NaCl showed a significant reduction in spikelets per spike, delayed spike emergence and reduced fertility, which results in poor grain yields. However, Na^+ and Cl^- concentrations in the shoot apex of these wheat plants were below 50 and 30 mM, respectively, which is too low to limit metabolic reactions (Munns and Rawson, 1999). Hence, the adverse effects of salinity may be attributed to the salt-stress effect on the cell cycle and differentiation. Salinity arrests the cell cycle transiently by reducing the expression and activity of cyclins and cyclin-dependent kinases that results in fewer cells in the meristem, thus limiting growth. The activity of cyclin-dependent kinase is diminished also by post-translational inhibition during salt stress. Recent reports also show that salinity adversely affects plant growth and development, hindering seed germination, seedling growth, enzyme activity (Seckin et al., 2009), DNA, RNA, protein synthesis and mitosis (Tabur and Demir, 2010; Javid et al., 2011).

4. Amelioration of salinity

Salinization can be restricted by leaching of salt from root zone, changed farm management practices and use of salt tolerant plants. Irrigated agriculture can be sustained by better irrigation practices such as adoption of partial root zone drying methodology, and drip or micro-jet irrigation to optimize use of water. The spread of dry land salinity can be contained by reducing the amount of water passing beyond the roots. This can be done by re-introducing deep rooted perennial plants that continue to grow and use water during the seasons that do not support annual crop plants. This may restore the balance between rainfall and water use, thus preventing rising water tables and the movement of salt to the soil surface (Manchanda and Garg, 2008). Farming systems can change to incorporate perennials in rotation with annual crops (phase farming), in mixed plantings (alley farming, intercropping), or in site-specific plantings (precision farming) (Munns et al., 2002). Although the use of these approaches to sustainable management can ameliorate yield reduction under salinity stress, implementation is often limited because of cost and availability of good water quality or water resource. Evolving efficient, low cost, easily adaptable methods for the abiotic stress management is a major challenge. Worldwide, extensive research is being carried out, to develop strategies to cope with abiotic stresses, through development of salt and drought tolerant varieties, shifting the crop calendars, resource management practices etc. (Venkateswarlu and Shanker, 2009) as shown in Fig. 1.

5. Use of salt tolerant crops and transgenics

Using the salt-tolerant crops is one of the most important strategies to solve the problem of salinity. Tolerance will be required for the “de-watering” species, but also for the annual crops to follow, as salt will be left in the soil when the water table is lowered. Salt tolerance in crops will also allow the more effective use of poor quality irrigation water. To increase the plant salt-tolerance, there is a need for understanding the mechanisms of salt limitation on plant growth and the mechanism of salt tolerance at the whole-plant, organelle, and molecular levels. Under saline conditions, there is a change in the pattern of gene expression, and both qualitative and quantitative changes in protein synthesis. Although it is generally agreed that salt stress brings about quantitative changes in protein synthesis, there is some controversy as to whether salinity activates specialized genes that are involved in salt stress. Salt tolerance does not appear to be conferred by unique gene(s) (Manchanda and Garg, 2008). When a plant is subjected to abiotic stress, a number of genes are turned on, resulting in increased levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection to these stresses (Bhatnagar-Mathur et al., 2008). Efforts to improve crop performance by transgenic approach under environmental stresses have not been that fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood.

Development of salt-tolerant crops has been a major objective of plant breeding programs for decades in order to maintain crop productivity in semiarid and saline lands. Although several salt-tolerant varieties have been released, the overall progress of traditional breeding has been slow and has not been successful as only few major determinant genetic traits of salt tolerance have been identified (Schubert et al., 2009; Dodd and Perez-Alfocea, 2012). 25 years ago Epstein et al. (1980) described the technical and biological constraints to solving the problem of salinity. Although there has been some success with technical solutions to the problem, the biological solutions have been more difficult to develop because a prerequisite for the development of salt tolerant crops is the identification of key genetic determinants of stress tolerance. The existence of salt-tolerant plants (halophytes) and differences in salt tolerance between genotypes within salt-sensitive plant species (glycophytes) indicates that there is a genetic basis to salt response (Yamaguchi and Blumwald, 2005). Although a lot of approaches have been done for development of salt tolerant plants by transgenics complete success is not achieved yet. The assessment of salt tolerance in transgenic experiments has been mostly carried out using a limited number of seedlings or mature plants in laboratory experiments. In most of the cases, the experiments were carried out in greenhouse conditions where the plants were not exposed to those conditions that prevail in high-salinity soils (e.g. alkaline soil pH, high diurnal temperatures, low humidity, and presence of other sodic salts and elevated concentrations of selenium and/or boron). The salt tolerance of the plants in the field needs to be evaluated and, more importantly, salt tolerance needs to be evaluated as a function of yield. The evaluation of field performance under salt stress is difficult because of the variability of salt levels in field conditions (Richards, 1983) and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity and water loss due to transpiration. Evaluating tolerance is also made more complex because of variation in sensitivity to salt during the life cycle. For example, in rice, grain yield is much more affected by salinity than in vegetative growth (Khatun and Flowers, 1995). In tomato, the ability of the plants to germinate under conditions of high salinity is not always correlated with the ability of the plant to grow under salt stress because both are controlled by different mechanisms (Foolad and Lin, 1997), although some genotypes might display similar tolerance at germination and during vegetative growth (Foolad and Chen, 1999). Therefore, the assessment of stress tolerance in the laboratory often has little correlation to tolerance in the field. Although there have been many successes in developing stress-tolerant transgenics in model plants such as tobacco, *Arabidopsis* or rice (Grover et al., 2003), there is an urgent need to test these successes in other crops. There are several technical and financial challenges associated with transforming many of the crop plants, particularly the monocots. First, transformation of any monocot other than rice is still not routine and to develop a series of independent homozygous lines is costly, both in terms of money and time. Second, the stress tolerance screens will need to include a field component because many of the stress tolerance assays used by basic researchers involve using nutrient-rich media (which in some cases include sucrose). This type of screen is unlikely to have a relationship to field performance. Third, because saline soils are often complex and can include NaCl, $CaCl_2$, $CaSO_4$, Na_2SO_4, high boron concentrations and alkaline pH, plants that show particular promise will eventually have to be tested in all these environments (Joseph and Jini, 2010).

6. Microbes: abiotic stress alleviation tool in crops

Several strategies have been developed in order to decrease the toxic effects caused by high salinity on plant growth, including plant genetic engineering (Wang et al., 2003), and recently the use of plant growth-promoting bacteria (PGPB) (Dimkpa et al., 2009). The role of microorganisms in plant growth promotion, nutrient management and disease control is well known and well established. These beneficial microorganisms colonize the rhizosphere/endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms (Nia et al., 2012; Ramadoss et al., 2013). Previous studies suggest that utilization of PGPB has become a promising alternative to alleviate plant stress caused by salinity (Yao et al., 2010) and the role of microbes in the management of biotic and abiotic stresses is gaining importance. The subject of PGPR elicited tolerance to abiotic stresses has been reviewed recently (Dodd and Perez-Alfocea, 2012; Yang et al., 2009).

The term Induced Systemic Tolerance (IST) has been proposed for PGPR-induced physical and chemical changes that result in enhanced tolerance to abiotic stress. PGPR facilitate plant growth indirectly by reducing plant pathogens, or directly by facilitating the nutrient uptake through phytohormone production (e.g. auxin, cytokinin and gibberellins), by enzymatic lowering of plant ethylene levels and/or by production of siderophores (Kohler et al., 2006). It has been demonstrated that inoculations with AM (arbuscular mycorrhizal) fungi improves plant growth under salt stress (Cho et al., 2006). Kohler et al., 2006 demonstrated the beneficial effect of PGPR *Pseudomonas mendocina* strains on stabilization of soil aggregate. The three PGPR isolates *P. alcaligenes* PsA15, *Bacillus polymyxa* BcP26 and *Mycobacterium phlei* MbP18 were able to tolerate high temperatures and salt concentrations and thus confer on them potential competitive advantage to survive in arid and saline soils such as calcisol (Egamberdiyeva, 2007). Kohler et al., 2009 investigated the

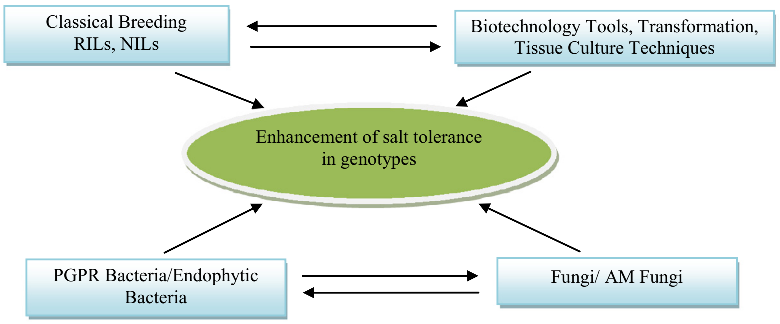

Figure 1 Different approaches for improvement of salt tolerance in agricultural crops.

influence of inoculation with a PGPR, *P. mendocina*, alone or in combination with an AM fungus, *Glomus intraradices* or *G. mosseae* on growth and nutrient uptake and other physiological activities of *Lactuca sativa* affected by salt stress. The plants inoculated with *P. mendocina* had significantly greater shoot biomass than the controls and it is suggested that inoculation with selected PGPR could be an effective tool for alleviating salinity stress in salt sensitive plants. Bacteria isolated from different stressed habitats possess stress tolerance capacity along with the plant growth-promoting traits and therefore are potential candidates for seed bacterization. When inoculated with these isolates, plants show enhanced root and shoot length, biomass, and biochemical levels such as chlorophyll, carotenoids, and protein (Tiwari et al., 2011). Investigations on interaction of PGPR with other microbes and their effect on the physiological response of crop plants under different soil salinity regimes are still in incipient stage. Inoculations with selected PGPR and other microbes could serve as the potential tool for alleviating salinity stress in salt sensitive crops. Therefore, an extensive investigation is needed in this area, and the use of PGPR and other symbiotic microorganisms, can be useful in developing strategies to facilitate sustainable agriculture in saline soils.

7. Alleviation of abiotic stress in plants by rhizospheric bacteria

Besides developing mechanisms for stress tolerance, microorganisms can also impart some degree of tolerance to plants towards abiotic stresses like drought, chilling injury, *salinity*, metal toxicity and high temperature. In the last decade, bacteria belonging to different genera including *Rhizobium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Burkholderia*, *Achromobacter*, *Azospirillum*, *Microbacterium*, *Methylobacterium*, *Variovorax*, *Enterobacter* etc. have been reported to provide tolerance to host plants under different abiotic stress environments (Grover et al., 2011). Use of these microorganisms per se can alleviate stresses in agriculture thus opening a new and emerging application of microorganisms. Microbial elicited stress tolerance in plants may be due to a variety of mechanisms proposed from time to time based on studies done. Production of indole acetic acid, gibberellins and some unknown determinants by PGPR, results in increased root length, root surface area and number of root tips, leading to an enhanced uptake of nutrients thereby improving plant health under stress conditions (Egamberdieva and Kucharova, 2009). Plant growth promoting bacteria have been found to improve growth of tomato, pepper, canola, bean and lettuce under saline conditions (Barassi et al., 2006; Yildirim and Taylor, 2005).

Some PGPR strains produce cytokinin and antioxidants, which result in abscisic acid (ABA) accumulation and degradation of reactive oxygen species. High activities of antioxidant enzymes are linked with oxidative stress tolerance (Stajner et al., 1997). Another PGPR strain, *Achromobacter piechaudii* ARV8 which produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase, conferred IST against drought and salt in pepper and tomato (Mayak et al., 2004). Many aspects of plant life are regulated by ethylene levels and the biosynthesis of ethylene is subjected to tight regulation, involving transcriptional and post-transcriptional factors regulated by environmental cues, including biotic and abiotic stresses (Hardoim et al., 2008). Under stress conditions, the plant hormone ethylene endogenously regulates plant homoeostasis and results in reduced root and shoot growth. In the presence of ACC deaminase producing bacteria, plant ACC is sequestered and degraded by bacterial cells to supply nitrogen and energy. Furthermore, by removing ACC, the bacteria reduce the deleterious effect of ethylene, ameliorating stress and promoting plant growth (Glick, 2007). The complex and dynamic interactions among microorganisms, roots, soil and water in the rhizosphere induce changes in physicochemical and structural properties of the soil (Haynes and Swift, 1990). Microbial polysaccharides can bind soil particles to form microaggregates and macroaggregates. Plant roots and fungal hyphae fit in the pores between microaggregates and thus stabilize macroaggregates. Plants treated with Exo-poly saccharides (EPS) producing bacteria display increased resistance to water and salinity stress due to improved soil structure (Sandhya et al., 2009). EPS can also bind to cations including Na^+ thus making it unavailable to plants under saline conditions. Chen et al., 2007 correlated proline accumulation with drought and salt tolerance in plants. Introduction of proBA genes derived from *B. subtilis* into *A. thaliana* resulted in production of higher levels of free proline resulting in increased tolerance to osmotic stress in the transgenic plants. Increased production of proline along with decreased electrolyte leakage, maintenance of relative water content of leaves and selective uptake of K ions resulted in salt tolerance in *Zea mays* coinoculated with *Rhizobium* and *Pseudomonas* (Bano and Fatima, 2009). Rhizobacteria inhabiting the sites exposed to frequent stress conditions, are likely to be more adaptive or tolerant and may serve as better plant growth promoters under stressful conditions. Moreover Yao et al., 2010 reported that inoculation with *P. putida* Rs 198 promoted cotton growth and germination under conditions of salt stress. Tank and Saraf (2010) showed that

Table 1 Role of plant growth promoting bacteria in salinity stress alleviation in plants.

Plant growth promoting bacterial species	Crop plant	Effect	References
Achromobacter piechaudii	Tomato (*Lycopersicon esculentum*)	Reduced levels of ethylene and improved plant growth	Mayak et al. (2004)
Azospirillum	Maize (*Zea mays*)	Restricted Na + uptake and increased K + and Ca$_2$ + uptake along with increased nitrate reductase and nitrogenase activity	Hamdia et al. (2004)
Aeromonas hydrophila/caviae Bacillus insolitus, *Bacillus* sp.	Wheat (*Triticum aestivum*)	Exopolysaccharide production	Ashraf (2004)
Pseudomonas syringae, *Pseudomonas fluorescens*, *Enterobacter aerogenes*	Maize (*Zea mays*)	ACC deaminase activity	Nadeem et al. (2007)
Pseudomonas fluorescens	Groundnut (*Arachis hypogea*)	Enhanced ACC deaminase activity	Saravanakumar and Samiyappan (2007)
Bacillus subtilis	Arabidopsis thaliana	Tissue specific regulation of sodium transporter HKT1	Zhang et al. (2008)
Pseudomonas mendocina	Lettuce (*L. sativa* L. cv. Tafalla)	ACC deaminase activity and enhanced uptake of essential nutrients	Kohler et al. (2009)
Rhizobium, *Pseudomonas*	Maize	Decreased electrolyte leakage and, increase in proline production, maintenance of relative water content of leaves, and selective uptake of K ion	Bano and Fatima (2009)
Pseudomonas pseudoalcaligenes, *Bacillus pumilus*	Rice (*Oryza sativa*)	Increased concentration of glycine betaine (compatible solute)	Jha et al. (2011)
Pseudomonas putida Rs-198	Cotton	Increase the absorption of the Mg^{2+}, K^{+} and Ca^{2+} and decrease the uptake of the Na^{2+} from the soil	Yao et al. (2010)
PGPR (Mk1, *Pseudomonas syringae; Mk20*, *Pseudomonas fluorescens*; and *Mk25*, *Pseudomonas fluorescens* biotype G) *and Rhizobium phaseoli* strains M1, M6, and M9	Mung bean	ACC deaminase activity and improvement in growth and nodulation in mung bean	Ahmad et al. (2011)
Raoultella planticola Rs-2	Cotton	ACC deaminase activity	Wu et al. (2012)
Brachybacterium saurashtrense (JG-06), *Brevibacterium casei* (JG-08), and *Haererohalobacter* (JG-11)	Groundnut (*Arachis hypogaea L.*)	High K^{+}/Na^{+} ratio and higher Ca^{2+}, phosphorus, and nitrogen content	Shukla et al. (2012)
Rhizobium phaseoli and PGPR (*Pseudomonas syringae*, Mk1; *Pseudomonas fluorescens*, Mk20 and *Pseudomonas fluorescens* Biotype G, Mk25)	Mung bean (*Vigna radiata* L.)	ACC deaminase activity and increased water use efficiency	Ahmad et al. (2012)
Rhizobium and Pseudomonas	Mung bean (*Vigna radiata* L.)	IAA production and ACC deaminase activity	Ahmad et al. (2013)
Pseudomonas putida, *Enterobacter cloacae*, *Serratia ficaria*, *and Pseudomonas* fluorescens	Wheat	Enhanced germination percentage, germination rate, and index and improved the nutrient status of the wheat plants	Nadeem et al. (2013)
Pseudomonas pseudoalcaligenes and Bacillus pumilus	Salt sensitive rice GJ-17	Reduce lipid peroxidation and superoxide dismutase activity	Jha and Subramanian, 2014
Acinetobacter spp. and Pseudomonas sp.	Barley and oats	Production of ACC deaminase and IAA	Chang et al. (2014)
Streptomyces sp. strain PGPA39	'Micro tom' tomato	ACC deaminase activity and IAA production and phosphate solubilization	Palaniyandi et al. (2014)

PGPRs which are able to solubilize phosphate, produce phytohormones and siderophores in salt condition promote growth of tomato plants under 2% NaCl stress.

In a study carried out by Naz et al., 2009, it was shown that strains isolated from Khewra salt range of Pakistan exhibited their tolerance when tested on saline media simulated by rhizosphere soil filtrate. Noteworthy, the isolates produced ABA in a concentration much higher than that of previous reports. Furthermore production of proline, shoot/root length, and dry weight was also higher in soybean plants inoculated with these isolates under induced salt stress. Likewise Upadhyay et al., 2011 studied the impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions and reported that co-inoculation with *B. subtilis* and *Arthrobacter sp.* could alleviate the adverse effects of soil salinity on wheat growth with an increase in dry biomass, total soluble sugars and proline content. Jha et al., 2011 reported that *P. pseudoalcaligenes*, an endophytic bacterium in combination with a rhizospheric *B. pumilus* in paddy was able to protect the plant from abiotic stress by induction of osmoprotectant and antioxidant proteins than by the rhizospheric or endophytic bacteria alone at early stages of growth. Plants inoculated with endophytic bacterium *P. pseudoalcaligenes* showed a significantly higher concentration of glycine betaine-like quaternary compounds and higher shoot biomass at lower salinity levels. While at higher salinity levels, a mixture of both *P. pseudoalcaligenes* and *B. pumilus* showed better response against the adverse effects of salinity. Nia et al., 2012 studied the effect of inoculation of *Azospirillum* strains isolated from saline or non-saline soil on yield and yield components of wheat in salinity and they observed that inoculation with the two isolates increased salinity tolerance of wheat plants; the saline-adapted isolate significantly increased shoot dry weight and grain yield under severe water salinity. The component of grain yield most affected by inoculation was grains per plant. Plants inoculated with saline-adapted *Azospirillum* strains had higher N concentrations at all water salinity levels.

Sadeghi et al., 2012 studied the plant growth promoting activity of an auxin and siderophore producing isolate of Streptomyces under saline soil conditions and reported increases in growth and development of wheat plant. They observed significant increases in germination rate, percentage and uniformity, shoot length and dry weight compared to the control. Applying the bacterial inocula increased the concentration of N, P, Fe and Mn in wheat shoots grown in normal and saline soil and thus concluded that Streptomyces isolate has potential to be utilized as biofertilizers in saline soils. More recently Ramadoss et al., 2013 studied the effect of five plant growth promoting halotolerant bacteria on wheat growth and found that inoculation of those halotolerant bacterial strains to ameliorate salt stress (80, 160 and 320 mM) in wheat seedlings produced an increase in root length of 71.7% in comparison with uninoculated positive controls. In particular, *Hallobacillus sp.* and *B. halodenitrificans* showed more than 90% increase in root elongation and 17.4% increase in dry weight when compared to uninoculated wheat seedlings at 320 mM NaCl stress indicating a significant reduction of the deleterious effects of NaCl. These results indicate that halotolerant bacteria isolated from saline environments have potential to enhance plant growth under saline stress through direct or indirect mechanisms and would be most appropriate as bioinoculants under such conditions. The isolation of indigenous microorganisms from the stress affected soils and screening on the basis of their stress tolerance and PGP traits may be useful in the rapid selection of efficient strains that could be used as bioinoculants for stressed crops. Some of the advances and researches carried out in evaluating role of rhizobacteria as salinity stress remediators have been summarized in Table 1.

8. Conclusion

An ideal sustainable agricultural system is one which maintains and improves human health, benefits producers and consumers both economically and spiritually, protects the environment, and produces enough food for an increasing world population. One of the most important constraints to agricultural production in world is abiotic stress conditions prevailing in the environment. Plant-associated microorganisms can play an important role in conferring resistance to abiotic stresses. These organisms could include rhizoplane, rhizosphere and endophytic bacteria and symbiotic fungi and operate through a variety of mechanisms like triggering osmotic response, providing growth hormones and nutrients, acting as biocontrol agents 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 expensive process, whereas microbial inoculation to alleviate stresses in plants could be a more cost effective environmental friendly option which could be available in a shorter time frame. Taking the current leads available, concerted future research is needed in this area, particularly on field evaluation and application of potential organisms as biofertilizers in stressed soil.

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ORIGINAL PAPER

Isolation and Characterization of *Streptomycetes* with Plant Growth Promoting Potential from Mangrove Ecosystem

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Abstract

A total of 66 actinomycetes isolates were isolated from mangroves of Andhra Pradesh, India, using various enrichment techniques and pre-treatments. The samples were collected from Coringa mangrove ecosystem and pre-treated by enrichment with $CaCO_3$, sodium dodecyl sulphate and phenol, plated on the media supplemented with cycloheximide (50 mg/ml), nystatin (25 mg/ml) and nalidixic acid (50 mg/ml). The population count of actinomycetes fluctuated from 1.9×10^5 to 8.0×10^5/g soil. Out of the isolated 66 actinomycetes, 8 isolates possessing plant growth promoting potential were further studied and characterized by physiological and biochemical traits and identified by 16S rRNA gene sequencing as different species of *Streptomycetes* genera.

Key words: 16S rRNA, ARDRA, IAA, mangroves, PGPR, *Streptomycetes*

Introduction

Mangrove is one of the most productive ecosystems and a natural renewable resource (Kathiresan, 2003; Balachandran *et al.*, 2009). These are a unique woody plant community of intertidal coasts in the tropical and subtropical zones, which are regarded as highly productive ecosystems and abode to unexplored microbial diversity including actinomycetes (Balagurunathan *et al.*, 2010). Mangroves provide a unique ecological niche to a variety of microorganisms (Agate, 1991). About 125 species of microorganisms (bacteria, fungi, algae) have been identified from mangrove environment (Kathirvel, 1996). Exploitation of less/unexplored ecosystems for actinomycetes is highly necessary for the discovery of novel bioactive metabolites (Sahoo and Dhal, 2009).

Among the different mangrove locations Andhra Pradesh of India has dense mangrove vegetation found towards coast rather than on shore land because of the dense branching network of creeks, which exist towards the coast (RSAM, 1992). There is more mangrove vegetation on tidal flats on the western side of the Krishna delta than on its eastern side. Dense mangroves are also seen over recent sand / mud spits on the Nizampatnam bay (RSAM, 1992). Sparse mangroves are found on the eastern side of the Krishna delta.

Actinomycetes being gram-positive bacteria showing a filamentous growth like fungi. They are aerobic and group of organisms widely spread in nature, with high G+C content (60–70 mol %) and are important sources of antibiotics (> 50%) and enzymes (Edwards, 1993; Gharaibeh *et al.*, 2003; Weber *et al.*, 2003; Shantikumar *et al.*, 2006).

Screening and isolation of promising actinomycetes from mangrove ecosystem with potential antimicrobial compounds is still a thrust area of research and it is suggested that the exploration of new areas and habitats played a vital role in the search for new microbes and novel metabolites (Horinouchi, 2002). The genus *Streptomyces* produce approximately 75% of commercially and medically useful antibiotics and 60% of antibiotics used in agriculture (Sanghvi *et al.*, 2014). Seasonal variations of antagonistic actinomycetes have been determined in selected mangrove ecosystems and highest numbers of actinomycetes during monsoon have been recorded. The microbial interrelationship in mangrove sediments does exist between bacteria and

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actinomycetes, bacteria and fungi, and fungi and actinomycetes (Rathna Kala, 1995). However, across the globe, the world's mangroves are threatened. Mangrove habitats are being destroyed as rivers are dammed, their waters diverted and the intertidal zone extensively developed for agriculture or aquaculture. Previous study showed that actinomycetes isolated from Malaysian soil have the potential to inhibit the growth of plant pathogens (Jeffrey *et al.*, 2007). Likewise actinomycetes isolated from Turkey's farming soil have the ability to inhibit *Erwinia amylovora* a bacteria that cause fireblight to apple and *Agrobacterium tumefaciens* a causal agent of Crown Gall disease (Oskay *et al.*, 2004). Besides acting agents for control of plant pathogens they also possess the capability for plant growth promotion (Nassar *et al.*, 2003; da Silva Sousa *et al.*, 2008). This is due to their capacity to produce IAA, antibiotics, siderophores, enzymes that have antimicrobial activity, substances that promote plant growth, solubilization of phosphates and competition with plant pathogens for substratum and nutrients (Hamdali *et al.*, 2008; da Silva Sousa *et al.*, 2008).

The present study aims at isolation of actinomycetes from mangrove ecosystem of Andhra Pradesh, India and to evaluate their plant growth promoting potential by siderophore and IAA production assay. The study also focusses on the genetic diversity study of the plant growth promoting isolates on the basis of morpho-physiological, biochemical and molecular characteristics.

Experimental

Materials and Methods

Soil sampling. A total of 10 soil samples and one water sample were collected from the Mangrove ecosystem of Kakinada District, Coringa (latitude 16°44′ to 16°53′N and longitude 82°14′ to 82°22′E), Andhra Pradesh, India by systematic sampling method. Samples were collected from 15 cm depth and transported to the laboratory in sterile bags and air-dried at room temperature. The geographical locations, as well as physiochemical properties of samples were recorded (Table I).

Isolation of actinomycetes. Samples were subjected to various enrichment techniques like $CaCO_3$ (Tsao *et al.*, 1960), SDS (Hayakawa and Nonomura, 1989), phenol (Hayakawa *et al.*, 2004), and media such as, Starch Casein Agar (SCA), Actinomycetes Isolation Agar (AIA) and Soil Extract Agar (SEA), amended with antibiotics nystatin (25 mg/ml), cycloheximide (50 mg/ml) and nalidixic acid (50 mg/ml), were employed for the isolation of *Streptomycetes*. The air dried samples were incubated at 55°C for 5 min in an incubator. One gram of soil was dissolved in 100 ml of distilled water (10^{-2} dilution) and 1 ml of 10^{-3}, 10^{-4}, 10^{-5} serial dilutions were spread plated on media amended with antibiotics using dilution-plate technique and incubated at 30°C for 2–3 weeks. After incubation, actinomycetes colonies were selected and maintained by sub-culturing on ISP-2 agar (g/l: 4.0 g glucose; 4.0 g yeast extract; 10.0 g malt extract; 18.0 g agar) slants and stored at 4°C for further use.

Identification by polyphasic taxonomy. Out of the isolated actinomycetes eight isolates (AM2-2, AM2-3, AM2-4, AM2-7, AM2-8, AM2-10, AM2-11, AM5-16) were characterized by morpho-physiological, biochemical and molecular methods. Identification of actinomycetes was carried out, using standard methods of morphological and physiological traits (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Williams *et al.*, 1989). Morphological methods consisted of macroscopic and microscopic studies. For chemotaxonomic studies, the colonies were grown in glucose yeast extract malt extract broth for 4–5 days and then filtered out and processed for determination

Table I

Details of sources of samples, pH and geographical Co-ordinates

Sample type	Source	Geographical Co-ordinates	pH	Cfu/g soil
Mangroves water sample	Nearby sea shore.	16°30”-17°N,82°10”-80°23”E	7.4	8.0×10^5
Rhizospheric soil	*Avicennia marina* (mangrove plant)	16° 56” N, 82° 13” E	7.2	2.25×10^5
Rhizospheric soil	*Avicennia officinalis* (mangrove plant)	16° 56” N, 82° 13” E	7.3	1.9×10^5
Rhizospheric soil	*Excoecaria agallocha* (mangrove plant)	16° 56” N, 82° 13” E	7.6	2.4×10^5
Rhizospheric soil	*Sonneratia apetala* (mangrove plant)	16° 56” N, 82° 13” E	7.2	3.06×10^5
Rhizospheric soil	*Aegicerale corniculatum* (mangrove plant)	16° 56” N, 82° 13” E	7.4	3.9×10^5
Rhizospheric soil	Grasses growing in mangrove	16° 56” N, 82° 13” E	7.0	2.86×10^5
Rhizospheric soil	*Ceriops decandra* (mangrove plant)	16° 56” N, 82° 13” E	7.2	3.93×10^5
Rhizospheric soil	*Rhizophora conjugata* (mangrove plant)	16° 56” N, 82° 13” E	7.5	3.61×10^5
Rhizospheric soil	*Hibiscus titraceous*	16° 56” N, 82° 13” E	7.1	4.05×10^5
Soil sample	Sample near the start of Coringa wild life sanctuary	16° 56” N, 82° 13” E	7.3	3.1×10^5

of the diaminopimelic acids (LL-DAP or meso-DAP) isomers and whole cell sugar patterns (Lechevalier and Lechevalier, 1980) using thin layer chromatography (TLC) on precoated cellulose plates (Merck, India).

Scanning electron microscopy. Spore surface ornamentation was evaluated by Scanning electron microscopy (SEM). Mycelia were taken (after 10 days of incubation) and washed in 0.1 M sodium cacodylate buffer (pH 7.4). They were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C followed by post-fixation with 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer (pH 7.4) and dried in a critical point dryer (EMITECH model K850, Hitachi). The preparations were mounted onto aluminium holders, sputter-coated with 10 nm Au and observed by SEM (Hitachi model S3400 at 15–30 kV, 2–5.00 µm).

Physiological characterization, plant growth promoting (PGP) attributes and extracellular enzymes production. Physiological characterization such as the effect of pH (6–9), temperature (20–45°C), salinity and carbon source utilization were studied. Carbohydrate utilization was determined by growth on carbon utilization medium (ISP-2) (Pridham and Gottlieb, 1948) supplemented with 1% carbon source (Glucose, Arabinose, Rhamnose, Mannitol, Dulcitol, Raffinose, Fructose, Sucrose, Lactose, Inositol). The intrinsic resistance of actinomycetes to salinity was evaluated according to Yadav *et al.* (2009), by observing the growth at 28 ± 2°C in tryptose soya broth amended with different concentrations of NaCl (2, 4, 6, 8% w/v). For growth promoting attributes, phosphate solubilization (Mehta and Nautiyal, 2001), siderophore production (You *et al.*, 2005), nitrate reduction (Glass *et al.*, 1997), cyanogenesis (Schippers *et al.*, 1990) and IAA production (Bano and Musarrat, 2003) were evaluated. The extracellular enzyme activity was assayed using standard methods such as amylase (Mishra and Behera, 2008), protease (Manachini *et al.*, 1988), cellulase (Farkas *et al.*, 1985), urease and gelatin degradation and hydrogen sulphide production were also studied following the protocol as reported by Cappuccino and Sherman (1992).

Molecular characterization

Extraction of actinomycetes DNA and PCR amplification. Genomic DNA was extracted from eight selected *Streptomycetes* isolates (having plant growth promoting properties) following the modified protocol of Boudjella *et al.* (2006). The purity check of DNA for analysing its quality and quantity was done by measuring the absorbance at 260 and 280 nm by spectrophotometer. The 16S rRNA gene fragment for the *Streptomycetes* was amplified by using two universal primer pair fD1 (5'-GAGTTTGATCCTGGCTCA-3') and Rp2 (5'-CGGCTACCTTGTTACGACTT-3'). The 16S rRNA was amplified by PCR using Promega kit. The final volume of reaction mixture of 50 µl contained 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1 mM of each primer, 0.25 U of *Taq* polymerase and 500 ng of template DNA. The amplification was performed on BioRad thermal cycler (initial denaturation step at 98°C for 3 min, after which *Taq* polymerase was added, followed by 30 amplification cycles of 94 C for 1 min, 52°C for 1 min, and 72°C for 10 min).

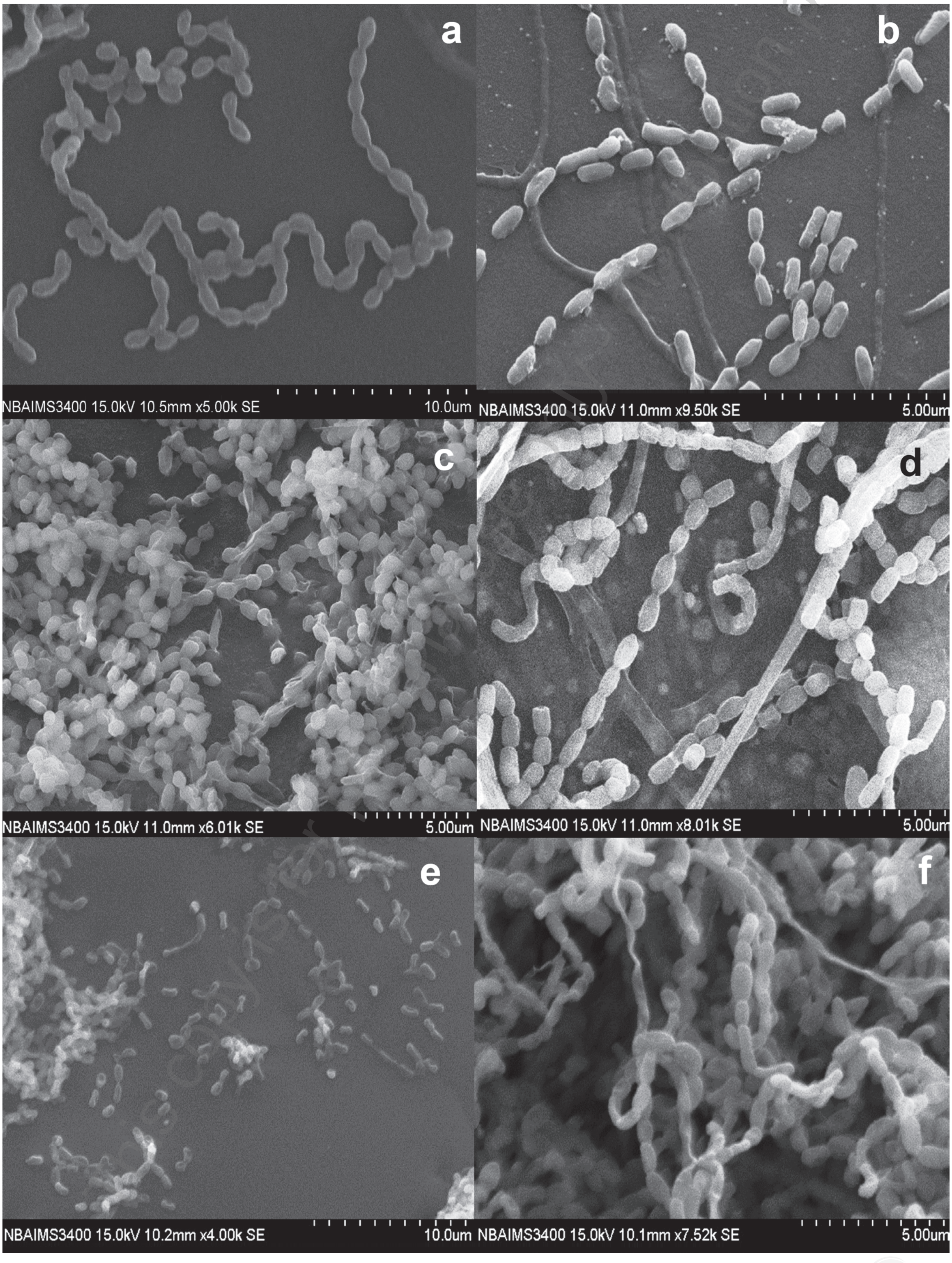
Amplified ribosomal DNA restriction analysis (ARDRA). For restriction analysis, amplified ribosomal DNA was digested with three restriction endonucleases *Taq* I, *Msp* I and *Hae* III (Promega, India) according to manufacturer's instructions and analyzed by horizontal electrophoresis in 2.5% agarose gels at 70 V for 2.5 h and documented on alpha-Imager gel documentation system (Alpha-Imager, USA). The restriction analysis profiles generated, were compared by calculating Jaccard's similarity coefficient for each pairwise comparison and dendrogram was constructed from the similarity matrix by the UPGMA. In order to test the goodness of fit of cluster analysis, co-phenetic value matrices were calculated and compared with the original similarity matrices that were UPGMA clustered by using the NTSYSpc analysis package (version 2.02e; Exeter Software, Setauket, NY). The amplified product of representative isolates from each clusters were purified by PCR purification kit (Promega, India) and sequenced directly with the Taq-mediated dideoxy chain terminator cycle sequencing in ABI 3130xl automated genetic analyser (Applied Biosystem, UK) according to manufacturer's instructions. The sequences were aligned by ClustalW and BlastN programme was used to compare the sequences deposited in public databases and the phylogenetic tree was constructed with the MEGA software version 4.1 (Saitou and Nei, 1987). Gaps were treated by pairwise deletions and bootstrap analysis was done by using 5,000 pseudo-replications.

Accession numbers: A total of eight sequences of 16S rRNA gene were deposited in public databases (GenBank, NCBI) under the accession numbers from KC511801 to KC511808.

Results

Isolation and clustering of actinomycetes by polyphasic taxonomy. A total of 66 actinomycetes isolates were isolated from Coringa mangrove ecosystem, of these eight isolates showed considerable amount of variations in their colony morphology and possessing plant growth promoting attributes were chosen for further physiological and biochemical characterization. The population count of actinomycetes fluctuated from 19×10^4 to 80×10^4 g soil^{-1} (Table I). Most of the isolates belonged to genus *Streptomycetes* and were tentatively identified by morphological characterization using aerial mycelial colour, substrate mycelial colour, pigments, arrangement of spores in chain, like straight chain, rectiflexibles *etc*. as revealed by scanning electron microscopy (Fig. 1). Cell wall composition analysis of actinomycetes using thin layer chromatography (TLC) revealed type I cell wall with LL-DAP isomers.

Physiological characterization of the isolated actinomycetes. Physiological tests are an important tool for classification of actinomycetes, influencing their growth and other properties. Physiological parameters like pH, temperature, NaCl concentration, carbon source utilization in the growth media were analysed. Growth of the selected 8 *Streptomycetes* isolates occurred in the pH range of 6–9 with optimum growth at pH 7.



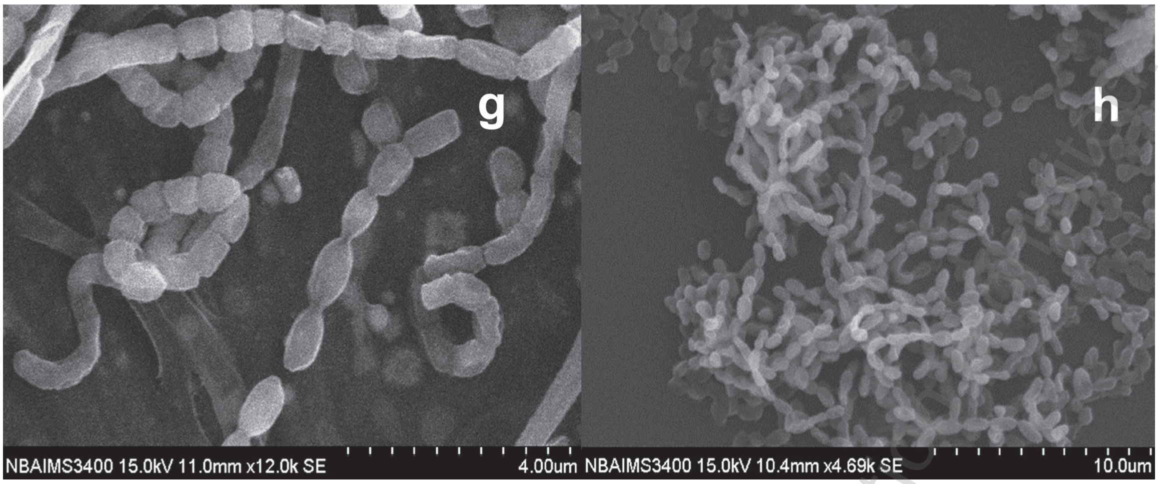


Fig. 1. Scanning electron microscopy (SEM) of *Streptomyces* isolates isolated from mangrove of Andhra Pradesh, India, showing variations in spore chain morphology.

a) AM2-2 (*Streptomyces globisporus*), (b) AM2-3 (*S. roseoviolaceus*), (c) AM2-4 (*S. cavourensis*), (d) AM2-10 (*S. celluloflavus*), (e) AM2-11 (*S. albogriseolus*) (f) AM2-7 (*S. spiralis*), (g) AM2-10 (*S. macrosporeus*) (h) AM5-16 (*S. rochei*)

The temperature range for growth was 20–45°C with the optimum temperature being 35°C. The isolates exhibited salt tolerance up to 8% with optimum growth at 6% NaCl; hence, the isolates could be placed in intermediate salt tolerance group. All the isolates were able to utilize 8–10 sugars as a carbon source out of 10 sugars being tested (Glucose, arabinose, rhamnose, mannitol, dulcitol, raffinose, fructose, sucrose, lactose and inositol).

Plant growth promoting attributes and extracellular enzyme production. Plant growth promoting and extracellular enzyme activity of the selected eight isolates revealed that all the 8 isolates (100%) were siderophore and IAA producers, 4 (50%) were phosphate solubilizers, 3 (37.5%) were H_2S and amylase producers, 5 (62.5%) were urease, protease and HCN producers, 6 75%) of the selected isolates were nitrate reducing and only 1 (12.5%) isolate produced the enzyme gelatinase (Table II).

Molecular characterization and phylogenetic analysis. The molecular characterization of the selected *Streptomycetes* isolated from mangroves were carried, based upon 16S rRNA gene amplification and its RFLP pattern with a set of three restriction enzymes *Taq*I, *Msp*I and *Hae*III (Fig. 2) and dendrogram (Fig. 3) was generated using NTSYSpc software. For More precise identification the isolates were sequenced by Sanger's di-deoxy nucleotide sequencing method and identified based on percentage similarity (>97% compared with public database sequences, NCBI), by BLAST homology (Table III). Further phylogenetic analysis of the isolates was carried out for their similarity to known actinobacteria aligned together with the sequences (closest representatives), available in public databases (GenBank, NCBI), of actinobacteria (Fig. 4). Three genetic groups were formed among the identified isolates (Table III). The 4 isolates included in group I showed 100% sequence identity compared with most closely related sequences in public database (*Streptomyces cavourensis*, *Streptomyces albogriseolus*, *Streptomyces spiralis*, *Streptomyces rochei*), followed by 2 isolates in group II with 99% similarity (*Streptomyces roseoviolaceus*, *Streptomyces celluloflavus*) and 2 isolates in group III showing <99% similarity (*Streptomyces globisporus*, *Streptomyces macrosporeus*).

Discussion

Actinomycetes population have been identified as one of the prominent group of soil microbes which differ with soil type, soil pH, geographical location and climatic condition (Arifuzzaman *et al.*, 2010). The characterization of these microbes is as important as studying their existence in the natural environments (Hirsch and Valdes, 2010). Actinomycetes play a vital role in the soil such as mineralization of organic matters, immobilization of nutrients, antibiosis and production of plant growth promoters (Adegboye and Babalola, 2012). In our studies heat pre-treatments, enrichment techniques and selective isolation media resulted in considerable decrease in the unwanted bacterial population, which was similar as obtained by Hayakawa (2008). By various enrichment techniques and media used a total of 66 actinomycetes were isolated out of which 8 isolates with significant plant growth promotory attributes and growth under different pH and salinity levels, were characterised by 16S rRNA sequencing as belonging to *Streptomycetes* genera. This genus had also been previously evaluated for the characterization of plant growth promoting and other important traits as

Table II
Phenotypic characteristics of all selected *Streptomycetes* strains.

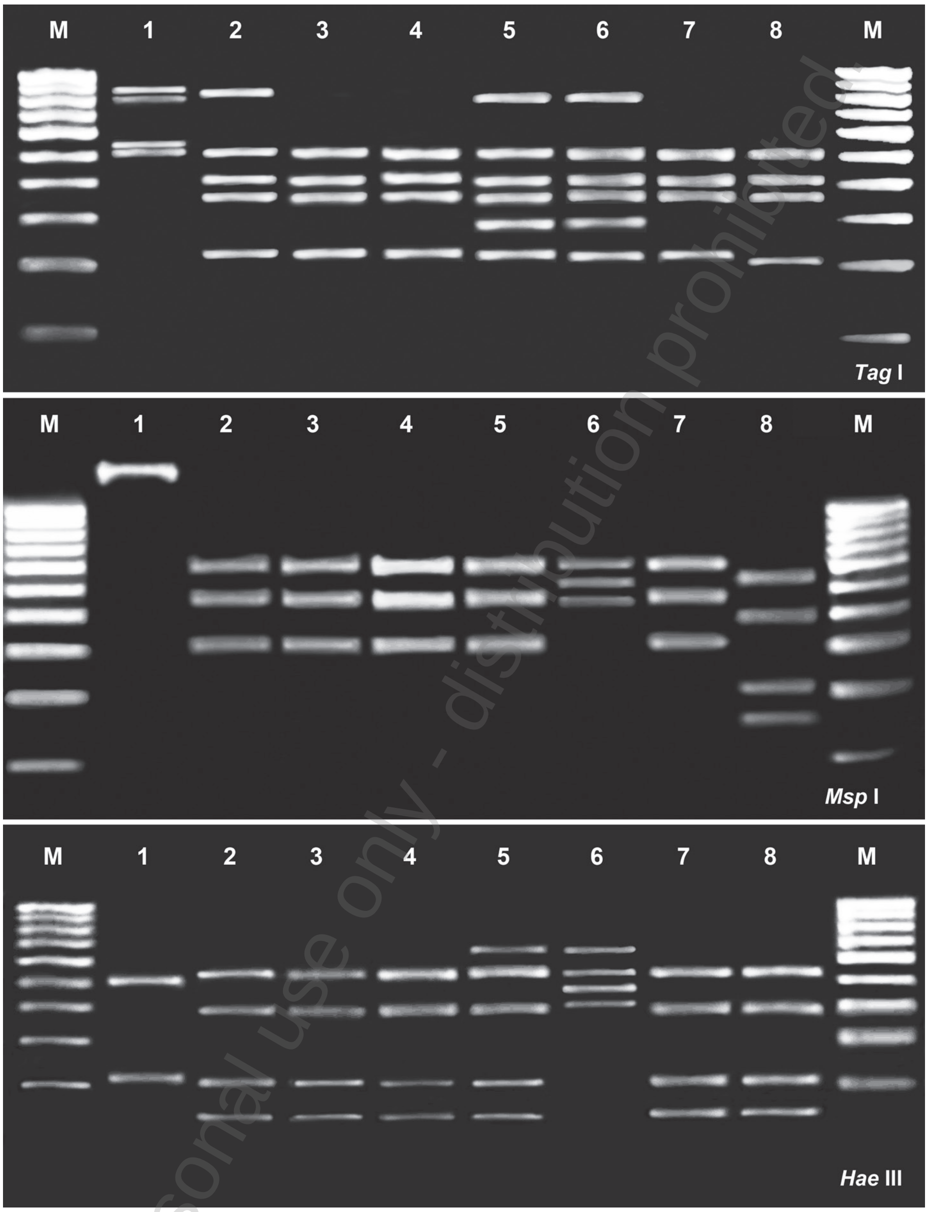
Properties	*S. globisporus* AM2-2	*S. roseoviolaceus* AM2-3	*S. cavourensis* AM2-4	*S. celluloflavus* AM2-10	*S. albogriseolus* AM2-11	*S. spiralis* AM2-7	*S. macrosporeus* AM2-8	*S. rochei* AM5-16
Morphological Characteristics								
Color of aerial mycelium	White	white	Ivory	White	White	White	White	Creamish white
Color of substrate mycelium	Yellow	Dark brown	Yellow brown	Light yellow	Yellow brown	Cream	Red brown	Yellow
Spore mass	Light brown	White	Ivory	Off white	Light grey	Cream	Creamish	Cream
Diffusible pigments	–	–	–	–	–	–	–	–
Carbon Source Utilization								
Glucose	+	+	+	–	+	+	+	+
Arabinose	–	+	+	+	–	+	+	+
Rhamnose	+	+	+	+	+	+	+	–
Mannitol	+	+	+	–	+	+	+	–
Dulcitol	–	+	+	+	–	+	+	+
Raffinose	+	+	+	+	+	+	+	+
Fructose	+	+	–	–	–	+	–	–
Sucrose	+	+	+	–	–	–	+	+
Lactose	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+
Chemotaxonomic characters								
Cell wall amino acid Analysis	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP
PGP traits								
IAA Production	+	+	+	+	+	+	+	+
Phosphate Solubilization	–	+	+	–	–	+	–	+
Siderophore Production	+	+	+	+	+	+	+	+
HCN Production	–	+	–	–	+	+	+	+
Nitrate Reduction	+	+	–	+	+	–	+	+
Hydrolytic enzymes and biochemical characterization								
Urease	+	–	–	+	+	–	+	+
Protease	–	+	–	+	+	–	+	+
Gelatinase	–	–	+	–	–	–	–	–
Amylase	+	–	–	–	–	+	–	+
H_2S Production	–	–	+	+	–	–	+	–

Table III
Closest BLASTN matches for the full 16S rRNA sequences and their percentage similarity with the closest actinobacterial strains.

IsolateCode	Closest species	GenBank accessionnumber	Similarity (%)
AM2-2	*Streptomyces globisporus*	KC511801	98%
AM2-3	*Streptomyces roseoviolaceus*	KC511802	99%
AM2-4	*Streptomyces cavourensis*	KC511803	100%
AM2-10	*Streptomyces celluloflavus*	KC511804	99%
AM2-11	*Streptomyces albogriseolus*	KC511805	100%
AM2-7	*Streptomyces spiralis*	KC511806	100%
AM2-8	*Streptomyces macrosporeus*	KC511807	98%
AM5-16	*Streptomyces rochei*	KC511808	100%

Fig. 2. Restriction digestion banding pattern of isolates amplified by 16S rDNA and digested by *Taq* I, *Msp* I and *Hae* III restriction enzymes.

Lane M: 100bp molecular weight marker (Genei, Bangalore), Lane 1: AM2-2, Lane 2: AM2-3, Lane 3: AM2-4, Lane 4: AM2-10, Lane 5: AM2-11, Lane 6: AM2-7, Lane 7: AM2-8 and Lane 8: AM5-16



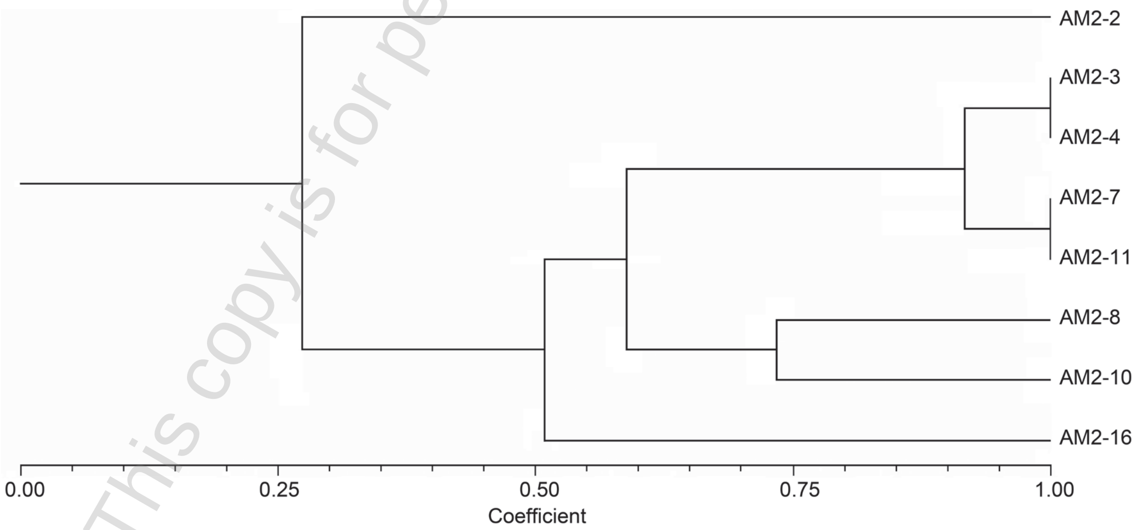


Fig. 3. UPGMA cluster analysis showing the genetic relationship among eight isolates of Actinomycetes, banding pattern based on restriction digestion by restriction enzyme *Taq* I, *Hae* III and *Msp* I

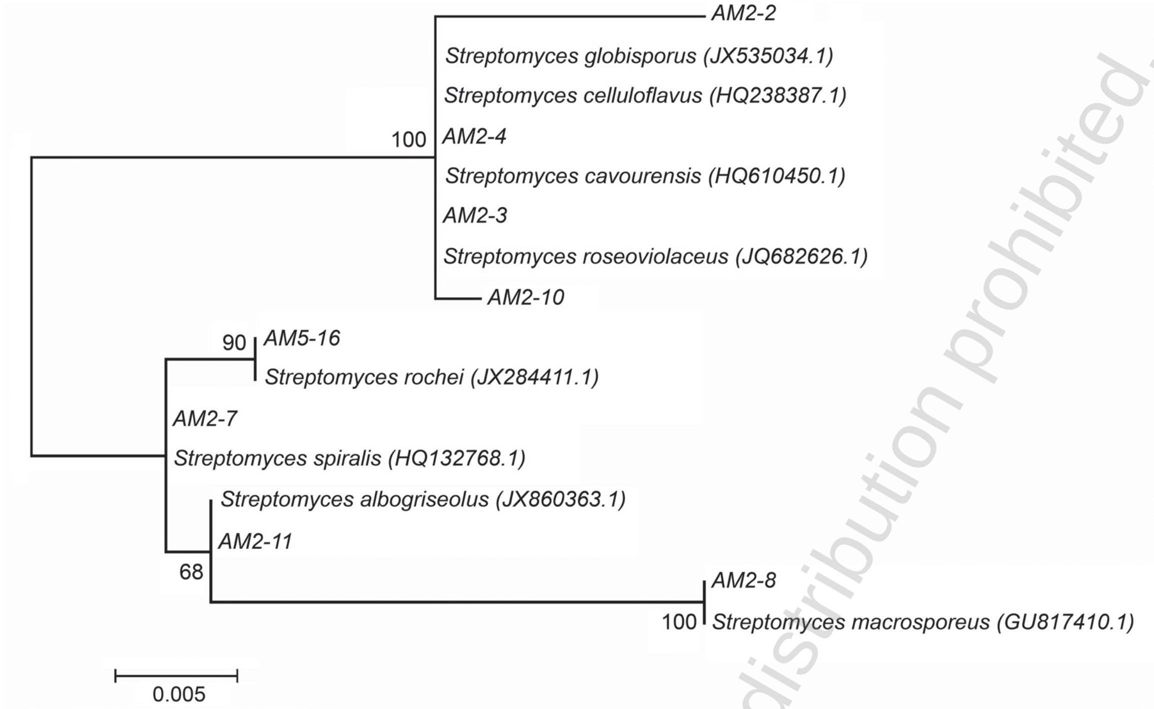


Fig. 4. NJ phylogenetic tree of full 16S rRNA sequences from selected isolates. The sequence data for several closely related actinobacterial type cultures were recovered from GenBank and included in the tree. The boot strap values from 5,000 pseudoreplications are shown at each of the branch points on the tree. Bar indicates % similarity

enough information available in the public databases (Zhang *et al.*, 1998; Malviya *et al.*, 2011; Yandigeri *et al.*, 2012), but *Streptomycetes* isolated from stressed habitats with plant growth promoting potential have not been studied extensively. Bacteria isolated from different stressed habitats possess stress tolerance capacity alongwith plant growth-promoting traits. Salinity tolerance and the ability of *Streptomycetes* for the production of extracellular enzymes, plant growth promoting attributes and other activities were evaluated and it was reported that all the 8 (100%) *Streptomycetes* isolates from mangrove region could tolerate up to 8% NaCl concentration showing optimum growth at 6% NaCl concentration, hence the strain could be placed in intermediate salt tolerance group according to Tresner *et al.* (1968). The ability of *Streptomyces* strains to tolerate high concentrations of NaCl is well known (Waksman, 1959). Similar results were reported from the *Streptomycetes* isolated from Indo-Gangetic Plains (IGP) (Malviya *et al.*, 2011), and halophilic *Streptomycetes* from India and their utilization in agricultural fields (Vasavada *et al.*, 2006). Da Silva Sousa *et al.* (2008) studied six *Streptomycetes* isolated from rhizospheric soil of various crops and found out that they could tolerate a NaCl level up to 3%. Kavya *et al.* (2012), also reported salinity tolerance of *Streptomycetes* from Coringa mangrove ecosystem but their results indicated a tolerance of 3–4% NaCl concentration, which is less as compared to isolates under current study. Sadeghi *et al.* (2012) have reported the beneficial role of *Streptomyces* on PGP activity under salinity stress. Hence, it can be concluded that these strains may have the ability to survive in the harsh environments such as saline and acidic to alkaline pH soils. In our studies all the 8 *Streptomycetes* isolates were positive IAA and siderophore producers. There are many reports which demonstrated the ability of endophytic and rhizospheric soil *Streptomycetes* to produce indole acetic acid and thus promote plant growth (Khamna *et al.*, 2010; Solans *et al.*, 2011; Yandigeri *et al.*, 2012; Kaur *et al.*, 2013). In the rhizosphere soils, root exudates are the natural source of tryptophan for rhizosphere micro-organisms, which may enhance auxin (IAA) biosynthesis in the rhizosphere. Likewise Sadeghi *et al.* (2012) have also reported the production of IAA and siderophore by halotolerant *Streptomycetes* isolate. Siderophore production may be involved in the inhibition of pathogens and thus promote plant growth indirectly because *Streptomyces* species are known for the production of hydroxamate type siderophores, which inhibit phytopathogen growth by competing for iron in rhizosphere soils (Khamna *et al.*, 2009; Kaur *et al.*, 2013), thus our isolates could be involved in the inhibition of pathogens. Malviya *et al.* (2011), also reported production of siderophore by *Streptomycetes* isolated from IGP region but in their studies only 8% of the isolates from the total 145 isolates were positive siderophore producers but in our studies 75% of the selected *Streptomycetes* were nitrate reducers, 50% were phosphate solubilizers and 62.5% were HCN producers. In agriculture, biological phosphate solubilisation as an alternative to natural phosphate utilisation plays an important role in efficient nutrient uptake of plants. Patil *et al.* (2010) isolated nine antagonistic actinomycetes from IGP region and found that out of them 8 were *Streptomycetes.* Out of these eight *Streptomycetes* isolates five were phosphate solubilizers and one produced HCN. Some actinomycetes are known to produce hydrolytic enzymes such as protease and amylase (Kaur *et al.*, 2013). These enzymes help in preventing the crops from plant pathogens and deleterious microbes by degrading their cell walls. In our study we reported that five of our isolates could produce enzyme protease and three isolates were amylase producers. Also it was reported that five isolates were producing urease, three H_2S producers and one isolate was able to show gelatinase activity. After studying the cultural and morphological characteristics of the potent plant growth promoting isolates, they were assigned under the genus *Streptomyces*. Concerning phenotypic characteristics, isolates produced varied colour aerial and substrate mycelia. Their chemotaxonomic characteristics further validate that they all belong to genus *Streptomyces* as their cell wall contain L-diaminopimelic acid (cell wall type-I). Thus, this study is in accordance with the previous reports that *Streptomycetes* are known to be predominant among actinomycetes with antagonistic and plant growth promoting potential and produce antifungal compounds (Ouhdouch *et al.*, 2001; Kaur *et al.*, 2013).

The reliable generic identification of members of this genus by the first approach was confirmed by DNA sequence analysis. Variations in 16S rDNA can also be assessed by analyzing restriction fragment length polymorphism (RFLP) of 16S rDNA sequences. PCR based markers such as RAPD, RFLP, DAPD and SSR have been used to discriminate bacterial strains and to analyse genetic diversity (Yadav *et al.*, 2013). In this study, we used 16S rDNA sequence-based phylogenetic analysis to investigate the species diversity of Streptomycetes. It is also known that microbes with 16S rRNA sequence similarity up to 97% identical should be considered as members of the same genus (Petit *et al.*, 1999; Malviya *et al.*, 2011). Since all the isolates were belonging to genus *Streptomyces*, we tried to study their diversity using ARDRA, which is the rapid and convenient method, and can be very useful in grouping actinobacterial isolates efficiently as well as effectively reduce the number of isolates by de-replicating the isolates during screening for diversity (Jiang *et al.*, 2010). In the current study, we used a set of three restriction endonucleases *Msp* I, *Hae* III and *Taq* I that specifically recognize the sequence 'CCGG', 'GGCC' and 'TCGA' respectively. The results of the different RFLP patterns obtained allowed us to effectively differentiate the strains into distinct groups of actinobacteria. In our study, a total of 8 representative isolates were identified by 16S rRNA gene sequence analysis as *S. globisporus* (KC511801), *S. roseoviolaceus* (KC511802), *S. cavourensis* (KC511803), *S. celluloflavus* (KC511804), *S. albogriseolus* (KC511805), *S. spiralis* (KC511806), *S. macrosporeus* (KC511807) and *S. rochei* (KC511808). All the isolates recorded 98–100% similarity with the representative isolates as shown in the Table III. Similar results were observed by Patil *et al.* (2010) and Malviya *et al.* (2011) the *Streptomyces* isolated by them also showed 98–100% similarity except one isolate in case of Malviya *et al.* 2011 which was 96% similar with the representative isolate.

The present study highlights that mangroves are the potential reservoirs for actinomycetes especially which can tolerate salt levels and biochemical and plant growth promotion assay of the isolates demonstrates their potential to be used as biocontrol agents and biofertilizers which may play important role in plant growth promotion either directly or indirectly and thereby increase crop yield.

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ORIGINAL ARTICLE

In vitro biocontrol activity of halotolerant *Streptomyces aureofaciens* K20: A potent antagonist against *Macrophomina phaseolina* (Tassi) Goid

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Abstract A halotolerant actinobacterial strain isolated from salinity affected soil of Eastern Indo-Gangetic plains (IGP), Uttar Pradesh, India, was characterised for its antagonistic potential against *Macrophomina phaseolina* by dual-culture assay. It was shown to effectively inhibit the growth of *M. phaseolina* with an inhibition zone of 27 ± 1.33 mm. Further the actinobacterial strain was evaluated for its plant growth promoting (PGP) properties and its ability to produce biocontrol related extracellular enzymes *viz.* amylase, protease, cellulase, chitinase, gelatinase and urease. The results revealed that the actinobacterial strain had PGP potential along with positive assay for amylase, chitinase and urease. The interaction study between antagonist strain and fungal pathogen, performed by scanning electron microscopy technique revealed that the actinobacterium was able to damage fungal mycelia may be due to chitinase, establishing its role as a potential antagonist against *M. phaseolina*. The actinobacterial isolate was characterised by 16S rDNA gene sequencing, and was identified as *Streptomyces* genera. The identified gene sequence was deposited to NCBI GenBank with an accession number KP331758.

1. Introduction

Fungal plant diseases are the major concern to worldwide agricultural production. The estimated yield loss due to plant diseases, range from 25% to 50% in the western countries and developing countries respectively (Gohel et al., 2006). Control of fungal diseases is imperative to ensure a constant food supply to an ever-increasing world population (Oskay, 2009; Evangelista-Martínez, 2014).

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The fungus *Macrophomina phaseolina* (Tassi) Goid is a soil-borne necrotrophic fungal pathogen that causes charcoal rot disease in over 500 different plant species, including various important crops *viz*. soybean, sorghum, maize, alfalfa and cotton. Charcoal rot has a wide geographical distribution, and the disease has been reported in tropical and subtropical regions of the world. Though, the fungus is primarily a soil-borne pathogen, in many crops it may be seed borne (Mah et al., 2012; Adhilakshmi et al., 2014). For decades, seed treatment with fungicides was recommended to control root rot of. *M. phaseolina*. It can be controlled effectively by fumigating the soil with methyl bromide; but the ever increasing cost of the application, restrictions on fungicide usage and concerns over environmental impact clearly indicate the need to search for effective control management strategies (Gopalakrishnan et al., 2011a). Research during the last two decades has led to the possibility of biological control as an increasingly realistic option for the management of plant pathogens. Biological control provides an environmentally safe and potentially stable alternative to chemical control. Several strains of actinomycetes have been found to protect plants against diseases through their potential to serve as: (a) source of agro-active compounds, (b) plant growth-promoting organisms and (c) biocontrol tools of plant diseases (Doumbou et al., 2001; Abdallah et al., 2013).

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal agents that inhibit several pathogenic fungi (Gopalakrishnan et al., 2011b; Al-Askar et al., 2014) and antibacterial agents (Arasu et al., 2013; Zhang et al., 2013). The antimicrobial mechanisms may involve physical contact (hyperparasitism), synthesis of hydrolytic enzymes, toxic compounds or antibiotics as well as competition (Taechowisan et al., 2005; Dhanasekaran et al., 2008). However, there is still considerable interest in finding more efficient strains, which differ considerably with respect to their antimicrobial effectiveness. The need for new, safe and more effective antifungal agents are a major challenge to the plant protection industry today since saline stressed habitats are potential reservoirs for bioactive actinobacteria (Jose and Jebakumar, 2014). The success of a good biocontrol agent requires more than one parameter *viz*. it should be effective for biocontrol over a long duration and survive under adverse conditions, and if the biocontrol agent can improve plant growth, it will be an added advantage. Therefore, the present study seeks to isolate halotolerant actinobacteria from saline habitat with antagonistic activity against the phytopathogenic fungus *M. phaseolina*, to identify the actinobacterium by 16S rDNA gene sequence analysis and to investigate for plant growth promotory attributes *in vitro*.

2. Materials and methodology

2.1. Isolation of actinobacteria and selection of halotolerant strains

Actinobacteria were isolated from composite soil samples collected from salinity affected regions of Eastern IGP, Uttar Pradesh, India (coordinates ranging from 25°93′ to 27°23′ N latitude and 79°02′ to 80°13′ E longitude). The pH of soil samples ranged from 7.4 to 10.8 and electrical conductivity (EC) from 2.24 to 13.26 dsm^{-1}. Isolation was done on starch casein agar (SCA) and actinomycetes isolation agar (AIA) media amended with cyclohexamide (50 $\mathrm{mg\,L}^{-1}$) to reduce fungal contamination (Labeda and Shearer, 1990) by standard serial dilution spread plate technique. Sample dilution plates were incubated at 32 ± 2 °C for 7–10 days until sporulated or non-sporulated actinomycete colonies were observed. Selected colonies were then inoculated onto yeast extract malt extract agar (ISP-2) for purification and stored at 4 °C in slant agar and in 20% glycerol at −20 °C. Screening of halotolerance capacity of isolated actinobacteria was evaluated by observing the growth in starch casein agar amended with various concentrations of NaCl (2–10%; w/v) along with control plates maintained with 0.5% NaCl (w/v) followed by incubation at 32 ± 2 °C for 5–7 days.

2.2. In vitro antagonistic bioassay

The twenty eight halotolerant actinobacterial isolates were evaluated for their activity towards pathogenic fungi *M. phaseolina* by dual-culture *in vitro* assay following the protocol of Khamna et al., (2009) with a slight modification. The fungal pathogen *M. phaseolina* used in the present study was isolated from root rot infected plants on potato dextrose agar (PDA; Himedia) medium and was maintained as pure cultures on PDA slants at 4 °C. The test isolates were streaked at one corner (1 cm from the edge) of the modified potato dextrose agar (PDA) (50% PDA + 50% SCA) plates (90 mm diameter) 48 h before pathogen inoculation. 5 day old fungal discs (8 mm diameter), grown on potato dextrose agar (PDA) at 28 °C were placed at another corner (1 cm from the edge) of modified PDA plates opposite to actinobacterial streak. Plates without the test isolate served as control. All plates were incubated at 30 ± 2 °C for 5 days. After incubation, the zone of inhibition (in mm) was measured and colony growth inhibition (%) was calculated by using the formula: $\mathrm{PI} = (C - T)/C \times 100$, where PI is the percent inhibition, C is the colony growth of pathogen in control, and T is the colony growth of pathogen in dual culture. All isolates were tested in triplicate.

2.3. Scanning electron microscopy to study interaction between promising isolate and fungal pathogen

The interaction of the test fungi with most promising antagonistic isolate (Actinobacterial strain K20) based on dual culture assay, was studied by scanning electron microscopy (SEM). The hyphae from the interaction zone was transferred on glass cover slips, then fixed with 1.5% glutaraldehyde and dehydrated with graded series of ethanol washes followed by drying in desiccator (Yuan and Crawford, 1995). Samples were affixed to SEM stubs using carbon tape followed by thin coating with gold: palladium (60:40) and examined by SEM (Hitachi model S3400).

2.4. Physiological and genotypic characterisation of the potent antagonist

Morphological and physiological characterisation of the potential antagonists was done on ISP-2 medium (10 g of malt extract powder, 4 g of yeast extract powder, 4 g of glucose and 20 g of agar in 1 l of distilled water) after 1 week of incubation. Sugar utilisation pattern was studied on basal medium [2.64 g of $(NH_4)_2SO_4$, 2.38 g of KH_2PO_4, 5.65 g of $K_2HPO{\cdot}3H_2O$, 1.0 g of $MgSO_4{\cdot}7H_2O$, 6.40 mg of $CuSO_4{\cdot}5H_2O$, 1.10 mg of $FeSO_4{\cdot}7H_2O$, 7.90 mg of $MnCl_2{\cdot}4H_2O$ and 15 g of agar in 1 l of distilled water and pH was adjusted to 6.8–7.0] (Promnuan et al., 2009) amended with various carbon sources (Table 1). For molecular identification, genomic DNA of isolate K20 was extracted through enzymatic lysis (Pospiech and Neumann, 1995) with little modification in lysozyme and proteinase K concentration. PCR (Peltier Thermal Cycler, BIORAD) amplification was performed using actinomycete specific 16S universal primers sets, ACT283F (5′-GGG TAG CCG GCC UGA GAG GG-3′) and 1360R (5′-CTG ATC TGC GAT TAC TAG CGA CTC C-3′) (McVeigh et al., 1996). The final volume of the 50 μl reaction mixture contained: 1X PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 100 μM of each dNTP, 1 pM of each primer, 0.25U of Taq polymerase and 50 ng of template DNA under the amplification condition as follows: initial denaturation at 98 °C for 3 min, followed by 30 amplification cycles of 94 °C for 60 s, 54 °C for 60 s, and 72 °C for 90 s. The actinobacterial strain K20 was directly using Taq-mediated dideoxy chain terminator cycle in ABI 3130xl automated genetic analyser (Applied Biosystems, UK) with same primer. The sequence was searched against BLASTn programme for its identity against the sequences deposited in public databases and the phylogenetic tree was constructed with the MEGA software version 4.1 (Saitou and Nei, 1987) with its related sequences. Gaps were treated by pairwise deletions and bootstrap analysis was done using 1000 pseudo replications.

2.5. Accession number

The identified 16S rDNA partial gene sequence of antagonistic strain (K20) was deposited at NCBI GenBank database under accession number KP331758.

2.6. In vitro plant growth promotory (PGP) attributes of actinobacterial strain K20

Actinobacterial isolate K20 was assayed for various PGP properties *in vitro viz.,* siderophore production was determined according to the methodology described by Schwyn and Neilands (1987). Antagonistic strain was streaked on Chrome Azurol S (CAS) agar media and incubated at 32 ± 2 °C for five days. When the actinomycetes consume the iron present

Table 1 Habitat, accession number, phenotypic characteristics, PGP traits and hydrolytic enzyme production by *S. aureofaciens* strain K20.

Characteristics		Observation
Habitat		Salinity affected soil of eastern IGP, Uttar Pradesh, India
GenBank accession number		KP331578
Morphological characteristics	Colour of substrate mycelia	Colourless to light cream
	Colour of spore mass	Greyish
	Diffusible pigment	–
	Motility	–
	Gram reaction	+
	Shape of spore chain	Rectiflexibilis
	Spore surface	
Cell wall amino acid analysis	Diaminopimelic acid (DAP)	L-DAP
Carbon source utilisation	Glucose	+
	Fructose	+
	Xylose	+
	Raffinose	+
	Cellobiose	+
	Arabinose	+
	Mannose	+
	Galactose	+
	Inositol	+
	Sucrose	+
Salt tolerance limit (% NaCl tolerance)	0–10%	+ (optimum at 8%)
Growth on different temperature	25–45 °C	+ (optimum at 35 °C)
Growth on different pH	7–8.5	+ (optimum at 7.5)
PGP traits and extracellular enzyme production		
	IAA production (μg mg^{-1} protein)	45.02
	Siderophore production	+
	Phosphate solubilisation	+
	Ammonia production	+
	HCN production	–
	Amylase production	+
	Protease production	–
	Cellulase production	–
	Chitinase production	+
	Gelatinase production	–
	Urease production	+

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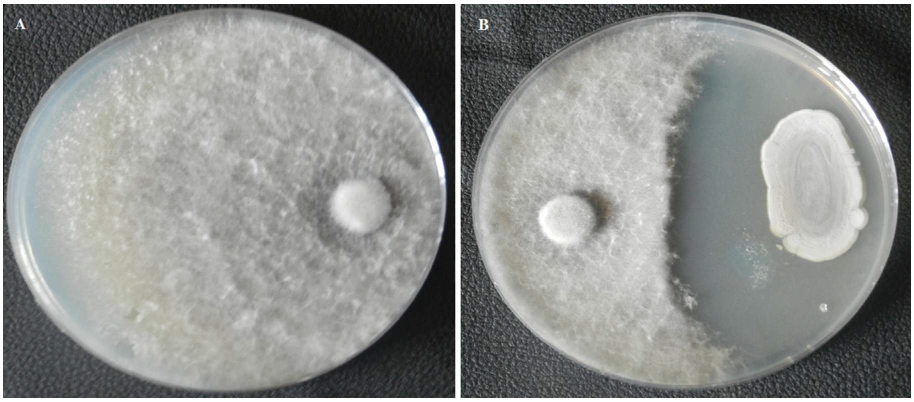

Figure 1 Micrographs for antifungal activity (dual culture assay) of *Streptomyces aureofaciens* strain K20 against *M. Phaseolina* (A) control, (B) dual culture plate.

in the blue-coloured CAS media, orange halos are produced around the colonies, which indicate the presence of siderophores. Indole acetic acid (IAA) production was assayed as per the protocol of Patten and Glick (1996). The strain was grown in Starch casein broth supplemented with L-tryptophan (1 μg ml^{-1}) for five days at 32 ± 2 °C. At the end of the incubation, the culture was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected. One ml of this supernatant was allowed to react with 2 ml of the Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) for 30 min. At the end of the incubation, development of pink colour indicates the presence of IAA. Quantification of IAA was done by measuring the absorbance in a spectrophotometer (Schimadzu, Japan) at 530 nm. For determining the phosphate solubilisation capacity, the actinomycete isolate was inoculated on the Pikovskaya agar containing tricalcium phosphate and incubated at optimum temperature for 5–7 days for the determination of a halo or clear zone formation around the colony, indicating the solubilisation of tricalcium phosphate (Mehta and Nautiyal, 2001). HCN was estimated qualitatively by sulphocyanate colorimetric method (Lorck, 1948). The isolate was grown on the Bennett agar amended with glycine (4.4 g l^{-1}). One sheet of Whatman filter paper no. 1 (9 cm diameter) was soaked in 1% picric acid (in 10% sodium carbonate; filter paper and picric acid were sterilised separately) for a minute and stuck underneath the Petri dish lids. The plate was sealed with parafilm and incubated at 32 ± 2 °C for five days. Development of reddish brown

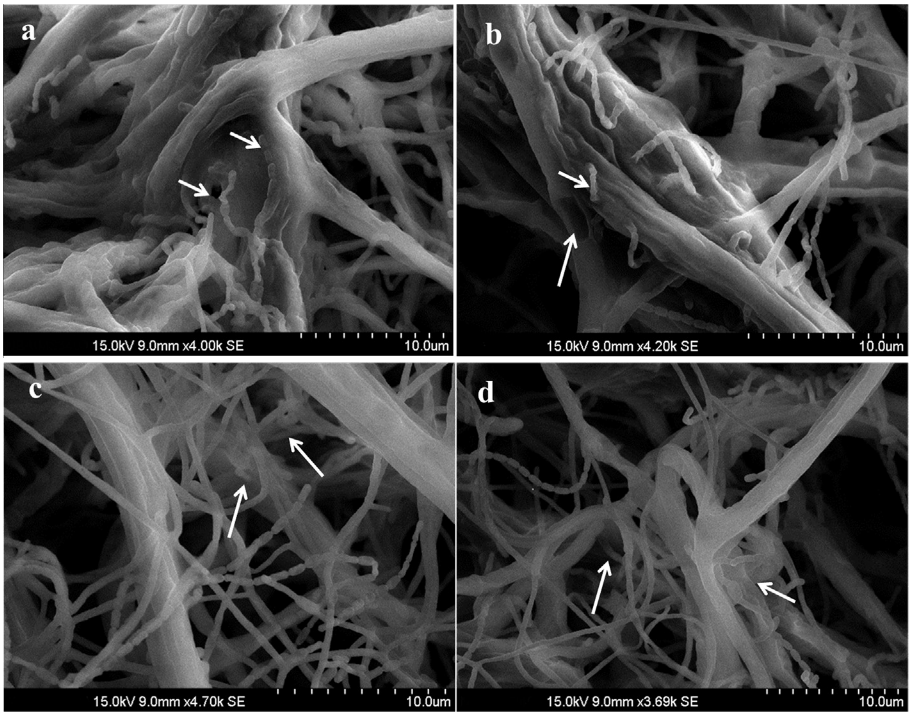

Figure 2 Scanning Electron Micrograph of *S. aureofaciens* strain K20 and the fungal pathogen *M. Phaseolina* after 5 days of interaction, (a–d) clustering of antagonist near fungal mycelia and causing destruction of the hyphae.

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colour on the filter paper is an indication for HCN production. Nitrate reduction test was done by inoculating freshly grown cultures in 10 mL peptone water broth in test tube and incubated for 48–72 h at 32 °C. Nessler's reagent (0.5 mL) was added to the test tube and the development of brown to yellow colour was treated as a positive test for ammonia production (Cappuccino and Sherman, 1992).

2.7. Extracellular enzymes production

The antagonistic halotolerant actinobacteria was screened qualitatively for the production of six important enzymes such as protease, chitinase, amylase, cellulase, gelatinase and urease. The qualitative assay for chitinase was performed on colloidal chitin agar (0.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.001 g of $ZnSO_4 \cdot 7H_2O$, 0.001 g of $MnCl_2 \cdot 7H_2O$, 1% glycerol, 5 ml of colloidal chitin in 1 l of distilled water and pH was adjusted 8.0 with 5 N NaOH). Colloidal chitin was prepared from crab shell chitin using concentrated HCl as described by Hsu and Lockwood (1975). Isolates were spot inoculated, then plates were incubated at 32 °C and clear halos around and beneath the growth, indicative of enzymatic degradation were observed after 5–7 days. Standard methods were followed for the assay of enzymes such as amylase (Mishra and Behera, 2008) protease (Manachini et al., 1988), cellulase (Farkas et al., 1985), gelatinase and urease (Cappuccino and Sherman, 1992).

3. Results

3.1. Isolation and selection of halotolerant actinobacterial isolates

In this study, fifty five actinobacterial isolates were isolated from the collected saline soil samples. The obtained isolates were screened for their salt tolerance potential. Among the fifty five actinobacterial isolates, twenty eight isolates were found to be halotolerant with salt tolerance capability of up to 10% NaCl (w/v). Out of these twenty eight halotolerant isolates screened for their antagonistic potential against *M. phaseolina* under a dual plate assay, only one isolate K20 efficiently inhibited the fungal growth, forming a large zone of inhibition and was therefore selected for further studies.

3.2. In vitro antagonism of isolate K20 and interaction studies

The actinobacterial isolate K20 strongly inhibited the growth of phytopathogen under dual culture assay (Fig. 1) with an inhibition zone of 27 ± 1.33 mm (Mean ± standard deviation) and percent inhibition of 64.52 ± 1.42%.

The *M. phaseolina* mycelia were obtained from the periphery of the inhibition zone of a 5 day old dual culture plate. Observations made under the scanning electron microscope revealed that the mycelia sample taken from dual culture assay plate was deformed, disintegrating and ruptured. As the antagonist entered the mycelial growth of fungal pathogen, it surrounded the mycelia growth completely and damaged it, reducing apical growth and irregular distortions in the fungal hyphae with reduction in the thickness of mycelia were observed (Fig. 2).

3.3. Morphological, physiological and genotypic characterisation of the isolate

The investigated antagonistic strain K20 formed an extensively branched substrate mycelium, aerial hyphae which carried smooth-surfaced spores in rectiflexibilis spore chains. It contained LL-diaminopimelic acid in the wall peptidoglycan, lacked characteristic major sugars and formed a greyish aerial spore mass on the starch casein agar (SCA) media and ISP-2 media with no soluble pigment formation which is a characteristic of the genus *Streptomyces*. Based on the physiological characterisation, the isolate was characterised as aerobic, mesophilic (growth between 25 and 45 °C with optimum at 35 °C), and moderately haloalkalitolerant (Salt tolerance up to 10% and pH tolerance range 7–8.5). Glucose, fructose, xylose, raffinose, cellobiose, arabinose, mannose, galactose, inositol and sucrose were used as sole carbon sources for energy and growth (Table 1). Based on the morphological and physiological characteristics, the isolate K20 was tentatively identified as belonging to genus *Streptomyces*. Assignment of the strain to the genus *Streptomyces* was confirmed

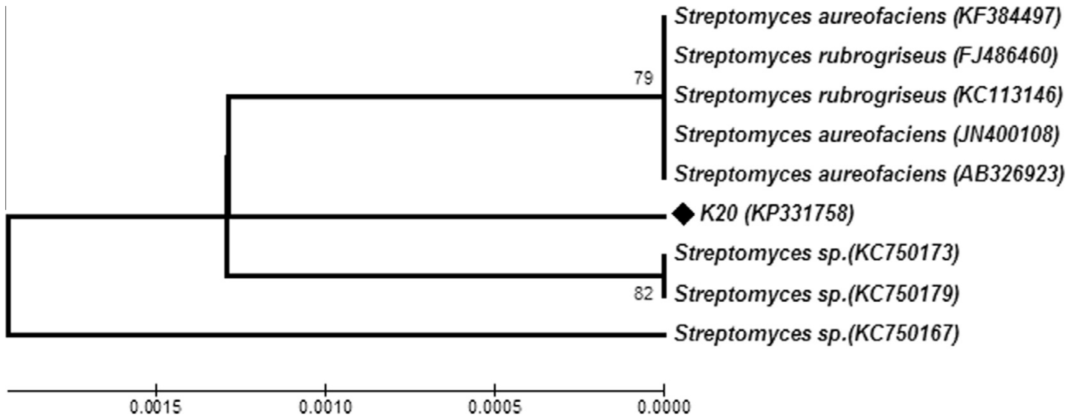

Figure 3 Phylogenetic tree based on the 16S rRNA gene sequences of halotolerant *Streptomyces* strain K20 and their closest phylogenetic relatives using UPGMA method. The bootstrap values from 1000 pseudo-replications are shown at each of the nodes. Bar indicates % similarity.

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by 16S rRNA gene sequencing studies. The 16S rRNA gene sequence of strain K20 was compared to the corresponding *Streptomyces* sequences already deposited in NCBI GenBank database, using BLASTn (NCBI website). The strain displayed 99% similarity with *S. aureofaciens*. 16S rRNA sequence of the strain K20 was deposited in public database (GenBank) under accession number KP331758. The *S. aureofaciens* strain K20 was grouped with other known *Streptomyces* species by UPGMA clustering (Fig. 3).

3.4. Plant growth promotory attributes and hydrolytic enzyme production

The study of plant growth promoting potential of the strain K20 under *in vitro* conditions revealed that the strain was capable of producing plant hormone IAA, siderophore (the iron chelating compound), and ammonia. The *Streptomyces* strain K20 was also solubilising phosphate but lacking the capacity to produce hydrogen cyanide (HCN). The amount of IAA produced by strain was 45.02 μg mg^{-1} protein. Among the tests performed for six important hydrolytic enzymes, the strain was positive for three enzymes i.e. amylase, urease and chitinase production while it tested negative for protease, cellulase and gelatinase production as shown in Table 1.

4. Discussion

The search for new natural products derived from microorganisms, especially from actinomycetes, has been focused on the isolation of species from unexplored and stressed niches to isolate novel or endemic species. These natural environments are still either unexplored or under-explored with adverse environmental conditions such as salinity and thus, can be considered as prolific resource areas for the isolation of diverse microorganisms. In the present study, 28 halotolerant actinobacterial strains isolated from salinity stressed habitat were assayed for antagonism against the phytopathogen *M. phaseolina*. One of the halotolerant isolate, *Streptomyces aureofaciens* K20 exhibited strong antifungal activity against the fungal plant pathogen, mainly attributed to the production of mycolytic enzymes, hyperparasitism and production of iron chelating compound siderophore. An appropriate screening system is important for selecting biocontrol agents. The screening system should be established according to the characteristics of the pathogen and the properties of the actinomycetes. Dual-culture assay has commonly been used for evaluating the antagonistic activity of actinomycetes against plant pathogenic fungi *in vitro* (Khamna et al., 2009; Kaur et al., 2013; Adhilakshmi et al., 2014). In this study, scanning electron microscopy was performed to find out the mode of action and interaction of the antagonist isolate K20 with the pathogen. The observations revealed that the strain K20 was capable of surrounding the fungus and degrading its mycelia further leading to holes in mycelia with its disruption. In a study done by Patil et al. (2010) on antagonism of *Streptomyces toxytricini* against fungal pathogen *Rhizoctinia solani* similar findings were reported under SEM interaction studies with the exception of hyperparasitism of antagonistic strain.

After studying the cultural and morphological characteristics of isolate K20 it was assigned under the genus *Streptomyces*. Concerning phenotypic characteristics, the isolate produced grey colour aerial and colourless to cream coloured substrate mycelia. According to microscopic morphological studies, the isolate formed rectiflexibilis type spore chain which bore smooth spores. The chemotaxonomic characteristics of the strain further validated that it belongs to genus Streptomyces as the cell wall contains L-diaminopimelic acid (cell wall type-I) and no characteristic sugar was detected in whole cell hydrolysate. Thus, this study is in accordance with the previous reports that Streptomycetes are known to be predominant among actinomycetes with antagonistic potential and produce antifungal compounds (Ramesh and Mathivanan, 2009; Khamna et al., 2009; Patil et al., 2010; Kaur et al., 2013; Rashad et al., 2015). The identity of the *Streptomyces* isolate was further confirmed by 16S rDNA sequence analysis, and isolate *Streptomyces* K20 revealed 99% sequence similarity with *Streptomyces aureofaciens*. The ribosomal gene (16S rDNA) sequencing has been used as a basic approach for the identification of microbial communities as well as for assessing microbial diversity in natural environments (Solanki et al., 2014).

It has been already established in several reports that microorganisms with stress tolerance capacity and plant growth promotion attributes are very helpful in growth and development of plants under stressed environments (Yandigeri et al., 2012; Shrivastava and Kumar, 2015). There are various mechanisms involved in the disease suppression, one of the primary mechanism of pathogen inhibition as used by plant growth promoting rhizobacteria (PGPR) includes the production of antibiotics, lytic enzymes, volatile compounds and siderophore (Harikrishnan et al., 2014). Our strain along with biocontrol capability exhibited plant growth promoting attributes (phosphate solubilisation, IAA, siderophore and ammonia production) and produced hydrolytic enzymes chitinase, amylase and urease. The halotolerant strain *S. aureofaciens* K20 was producing indole acetic acid (IAA) in a fairly good amount (45.05 μg mg^{-1} protein). There are many reports which demonstrate the ability of endophytic and rhizospheric soil streptomycetes to produce indole acetic acid and thus promote plant growth (Yandigeri et al., 2012; Harikrishnan et al., 2014). Khamna et al. (2009) and Gopalakrishnan et al. (2011a) have also reported the production of IAA by antagonistic actinobacteria isolated from medicinal plant rhizosphere and herbal vermicompost respectively. In agriculture, biological phosphate solubilisation as an alternative to natural phosphate utilisation plays an important role in efficient nutrient uptake and it was carried out by our strain in the present work and ammonia was also produced by *S. aureofaciens* K20. Similar results were reported by Kaur et al. (2013) in a study performed for evaluation of antagonistic and plant growth promoting activities of endophytic and soil actinobacteria. We reported the production of siderophore while HCN production was not seen in our antagonist strain K20. The data suggest that siderophore production may be involved in the inhibition of pathogen by the isolate and thus promote plant growth indirectly because *Streptomyces* species are known for the production of hydroxamate type siderophores, which inhibit phytopathogen growth by competing for iron in rhizospheric soils (Khamna et al., 2009; Gopalakrishnan et al., 2011a; Harikrishnan et al., 2014). *In vitro* evaluation showed that *S. aureofaciens* K20 produced lytic enzymes *viz.,* chitinase and amylase which might be responsible for strong antagonistic activity against fungal pathogen observed in our study. Chitinase production by antagonistic strain could be one of the possible mechanisms of biocontrol for fungal phytopathogens, because the cell wall of fungi consists of polysaccharides such as chitin and glucan (Patil et al., 2010; Kaur et al., 2013). Inhibition of *M. phaseolina* mycelial growth by K20 culture can be attributed to production of extracellular compounds such as cell wall degrading enzymes and antibiotics, which damaged and disintegrated the hyphae of pathogen as observed under SEM. The contact of strain K20 with pathogen resulted in the abnormalities such as distortion and hyphae destruction.

5. Conclusion

In the present scenario the search for new bioactive compounds has extended from medical field to agricultural field for crop protection and enhancing yields. The present study publicises that the saline stressed habitats are potential reservoirs for bioactive actinobacteria. *In vitro* antagonistic assay with our antagonist strain uncovers its potential to be utilised as biocontrol mechanism against phytopathogenic fungi *M. phaseolina*. The halotolerant Streptomyces strain K20 even possesses plant growth promotion potential by producing IAA, siderophore and ammonia and solubilises phosphate. Thus, directly as well as indirectly it can promote plant growth. So, it may be concluded that the strain *S. aureofaciens* K20 could be utilised for biocontrol management (*M. phaseolina*) programme in saline stressed soils.

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