ROLE OF PLANT GROWTH PROMOTING BACTERIA IN NUTRIENT ACQUISITION AND PLANT STRESS TOLERANCE RESPONSE IN SALINE SOIL

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Under the guidance of

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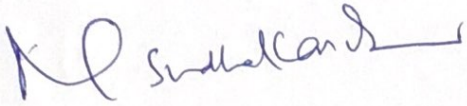
CERTIFICATE

Certified that the thesis "**ROLE OF PLANT GROWTH PROMOTING BACTERIA IN NUTRIENT ACQUISITION AND PLANT STRESS TOLERANCE RESPONSE IN SALINE SOIL**" which issubmitted by Miss ChetnaDewan, in the fulfilment of the requirement for the award of the degree of mater of science in biotechnology in the department of biotechnology (DBT), ThaparInstitute of Engineering and Technology, Patiala is a record of the candidate's own independent and original research work carried out by her under our guidance and supervision. The matter embodied in this thesis has not been submitted in part or full to any other Institute or University for the award of any degree.



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DECLARATION

I, ChetnaDewan hereby declare that the work which is being presented in the thesis "**ROLE OF PLANT GROWTH PROMOTING BACTERIA IN NUTRIENT ACQUISITION AND PLANT STRESS TOLERANCE RESPONSE IN SALINE SOIL"** submitted byme for the award of the degree of Master of Science in the department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is true and original record of my own independent and original research work carried out under the supervision of **Prof.Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology. The matter embodied inthis thesis has not been submitted in part or full to any other institute or university for the award of any degree in India or Abroad.

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ABSTRACT

High salt accumulation in soil is at increasing pace all over the world. It is caused by many natural as well as human activities, and causes degradation of soil structure in environment. Due to this, plant growth gets restricted in these areas because of nutrient deficiency. Many staple cereal crops are salt sensitive. This causes a reduction in their agricultural yield and people residing near these arid and semi arid regions, cannot get sufficient supply. Many microorganisms are not adaptable to grow in high salt concentration, and thus not able to release metabolites which promotes plant growth. Use of chemical fertilisers worsen the condition and contaminates the ecosystem. Therefore there is a need for biological way to resolve this problem.

The objective of the study was to isolate salt tolerant bacteria from natural saline habitats and to screen their plant growth promoting activities under salt stress. In the present study, two bacterial species were isolated from a Sambhar salt lake, Rajasthan. They were able to grow up to 20% salt concentrations, and produce sufficient amounts of plant growth promoting metabolites like IAA, ammonia, Siderophore which ultimately promotes plant growth. Both the isolates have the capacity to solublise phosphate, which was checked on Pikovskaya agar medium containing insoluble phosphate. Phosphorus is the most vital nutrient which plants require for their growth but less available to plants.

These isolates were used as inoculums on sorghum seeds for nursery experiment. A significant increase in plant growth, root length, shoots length, fresh & dry biomass was observed in treated plants under salt stress as compared to the control plant. Enzyme activities of soil were also quantified at the end of the experiment. Therefore these isolates can be potentially used for plant growth promotion in saline soils.

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INTRODUCTION

1.1 Soil salinization

Salinity stress is the world's biggest unfavourable environmental issue, which hampers many constraints globally. Salinity in soil is rising at a pace of 7% around the world (Tester *et al*., 2003). Nearly 20% of world's cultivated lands is affected by salinity these days (Glick *et al*., 2007). Soil salinity causes degradation of soil by affecting the nutrient content. Due to soil salinity, crop plants suffer from high osmotic stress, decreased nutritional value, thereby causing reduction in crop productivity and agricultural yield worldwide. When plants gets affected from germination stage to maturation stage by the excess amount of salt in soil, this adverse effect of excess salts accumulation is known as salt stress (Munns, 2005). Ionic, oxidative and osmotic stress are the secondary stress on plants due to high salt concentration. Salinity stress can affect biochemical pathways in plants by altering their enzyme activities.

There are many microbes which are present in soil, contributing to the fertility of soil by various enzyme activities. Due to high concentration of salts, microbial diversity gets inhibited leading to more acute effects on soil productivity (Tripathi *et al*., 2007). High ion concentration along with very high pH levels also limits microbial growth. Microbial growth and their enzyme activities also show negative correlation with increasing salinity in soil (Morrisey *et al*., 2014).

Salinity can be the caused by two types-

- Primary salt stress- It results from accumulation of excess salts through natural processes such as rock weathering, and release of some soluble salts of sodium, calcium, magnesium chlorides and less amount of sulphates and carbonate salts. It can also be triggered by deposition of some oceanic salts by rain and wind.
- Secondary salt stress – It is caused by many human activities that alter hydrological equilibrium of soil by improper irrigation practices (Garg and Manchanda, 2008). About 20% of degradation of soil by salts is caused by secondary salt stress (Glick *et al*., 2007).

Salinization is becoming a devastating threat to environment resources and human health in many countries. Over 7 million hectares of land is covered by the saline soil (Patel *et al*., 2011). In India a total of 6.7 Mha land is affected by salinity. Gujarat, Punjab, Uttar Pradesh, Haryana are the major states which come under this area. Some parts of Madhya Pradesh and Karnataka are also affected by salinity (Figure 1.1).

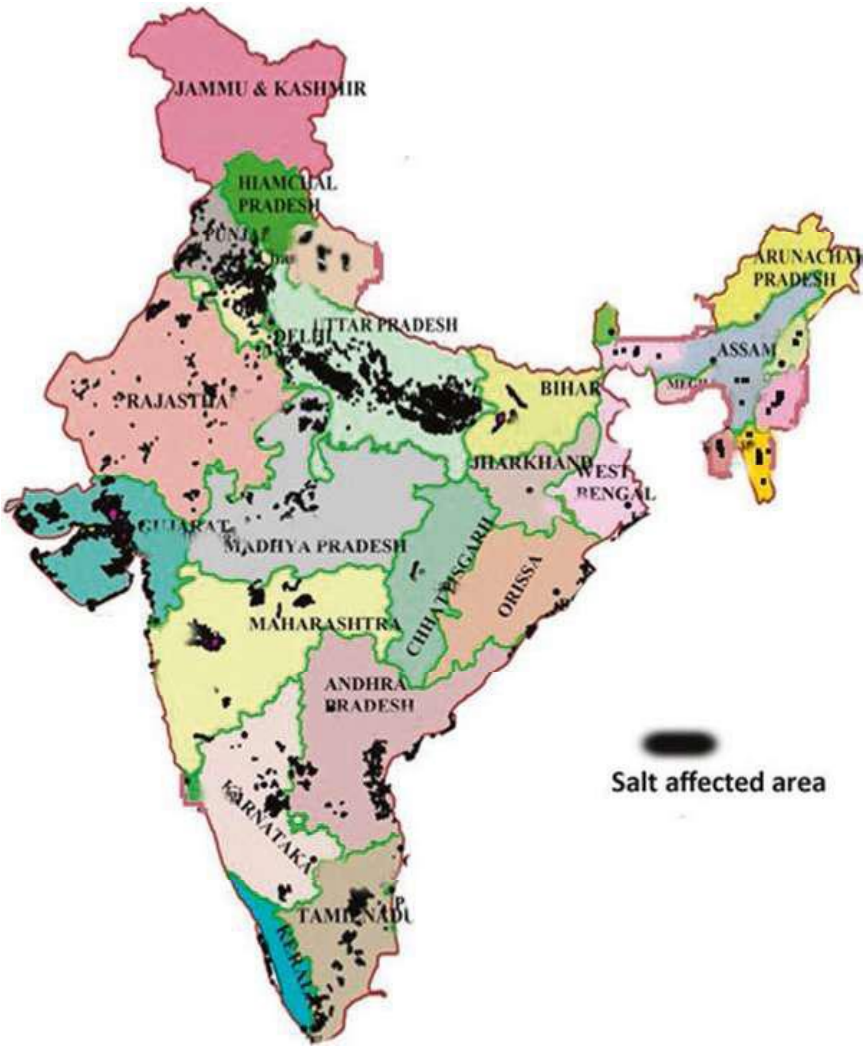


Figure 1.1 Distribution of saline areas in India (CSSRI, 2012).

Salinity affects plant growth mainly by two ways: either by ion toxicity or by limiting water uptake. It influences all phases of plant growth to some extent- germination, vegetative and reproductive. Plant length is affected due to reduced supply of phytohormones by salts accumulation. Due to salt stress Phosphorus uptake by plants also gets reduced because soluble phosphate ions gets precipitated with calcium ions and make it insoluble in soil (Bano and Fatima, 2009). Excessive sodium ions accumulation in cell walls of plants can lead to osmotic stress ultimately causing dehydration of cells and also cell death (Munns, 2003). Salinity also reduces leaf area and hence photosynthesis (Netondo *et al*., 2004). It also causes hormonal imbalance (Ashraf, 2004), which can lead to stunted growth. Salinity also inhibits microsporogenesis which can harm the reproductive development. Many enzyme activities decrease which affects the development of seedlings in many plants (Seckin *et al*., 2009). Reactive oxygen species and stress induced ethylene level increases under salt stress and causes reduction in agricultural yield.

Due to increased demand, producers become more reliable on chemicals to protect the crop from pests as well as enhance their yield. Phosphate is least available to soil after the harvest of the crop, which requires regular addition of phosphate by chemical means which is a costly affaire. Therefore there is a need to find a supplemented way to promote plant growth biologically, which is more effective.

Some microorganisms can be beneficial for plant growth. These microbes can colonise the roots of plant or can be free living. Plant growth promoting bacteria can promote plant growth by various direct and indirect mechanisms (Nia *et al*., 2012). Microorganisms that are able to live up and adapted to grow in the hypersaline regions are known as halophilic microorganisms (Zanjirband *et al*., 2013). Many of them belongs to *Actinobacteria*, *Firmicutes*, *Proteobacteria*. Bacteria that are isolated from hypersaline environment (more than 15% salt concentrations), that can grow under salt stress can be exploited for their plant growth promoting activities. Use of these microbes can act as a promising way to alleviate reduced growth of plants caused by salt stress (Yao *et al*., 2010).

The effect of a particular bacterial strain can differ with the conditions of plant and soil nutrients. Previously, only *Rhizobia* spp. were extensively used as plant growth promoting rhizobacteria but now many other bacteria which are free living , fix only less or no nitrogen can be used which show plant growth promotion by various other mechanisms. These bacteria can alter plant hormone levels under stress conditions (Glick *et al*., 2007).

Plant growth promoting bacteria-induced physical and chemical changes that result in enhanced tolerance to abiotic stress can be termed as induced systemic tolerance. Plant growth promoting bacteria facilitates plant growth indirectly by decreasing plant's contact with the pathogens present in soil (HCN production, siderophore production), or directly by production of some important phytohormones (auxin, cytokinin and gibberellins), by lowering plant ethylene levels through changes in enzymatic pathways and/or by production of siderophores (Kohler *et al*., 2009). Recently agriculturists are also attracted towards these soil inoculums for plant growth and better yield without any harm to their quality.

1.2 Objectives

- Isolation of halophilic bacteria from soil/sediment sample obtained from Sambhar salt lake
- Estimation of different plant growth promoting activities of halophilic bacterial isolates
- Evaluation of plant growth promotion using selected bacterial isolates as inoculums in nursery trial.

Chapter-2

REVIEW OF LITERATURE

There are many environmental stresses that limits in crop production as they affect plant growth and yield. Salt accumulation in soil due to precipitation of salts to a level which is up to cause toxicity, that harms plant growth is soil salinization (Shin *et al*., 2016). Recent studies showed that 5% of the total cultivated land is excess salt affected. Saline soil is present across all continents in the world, but extensively present in arid and semi arid regions and less in humid areas because of less rainfall in arid regions and transport of salts with water is less in the root zone area of plants. Presence of excess salts on the soil surface and in the root zone is the characteristic feature of saline soils. Microbial growth in the root zone area of plants growing in saline soils is much affected by salts in soil (Siddikee *et al*., 2011). Salt tolerant plants growing in these areas can be used in developing transgenic plants by breeding or plant genetic engineering methods. Other than these approaches, use of plant growth promoting bacteria which can withstand under stress conditions is a developing strategy (Egamberdieva *et al*., 2007).

2.1 Causes of soil salinity

The significance of affects of Soil Salinity on agricultural yield is very extensive (Tester, 2003). Other factors that increase salinity includes-

- Low rainfall
- Ineffective internal drainage
- Poor irrigation practices on cultivated land
- High evaporation
- Improper use of chemical fertilizers (Egamberdeva *et al*., 2007).

Currently, salinity in agriculture affects around 100 nations. Increasing salinity may cause 20 to 50 % decrease in the yields of important crops as compared to the record yields.

Based on the nature, characteristics and plant growth relationship in salt affected soils divided into two main types:

i. Saline soils- These soils contain soluble salts mainly sodium chloride and sodium sulphate in an adequate amount that can have negative impacts on plant growth and crop productivity. Saline soils may also contain soluble compounds of magnesium and cacium like gypsum.

ii. Sodic soils- Soils containing sodium salts which are capable of alkaline hydrolysis, mainly sodium carbonate. Sodic soils have large number of sodium salts that put negative charge on clay particles.

2.2 Measure of salinity

Soil salinity can be evaluated by passing an electrical current through the soil sample solution and is called Electrical Conductivity. Soil is considered as saline soil when the electrical conductivity of a saturated extract soil sample is 4-8 dSm^{-1} at room temperature. Above this range of EC, crop yield gets reduced to some extent (Shahbaz and Ashraf, 2013). The exchangeable sodium percentage of such soils is less than 15%. Because only Elecrical Conductivity cannot define the exact type/nature of the affected soil, pH is also taken as considerable measure for saline soils that is generally below 8.2. Electrical conductivity and microbial biomass in soil are also negatively correlated. Mavi *et al*., (2012) stated that salinity will decrease the soil microbial activity due to decreased organic matter decomposition. Table 2.1 shows electrical conductivity in a range has different effects on plants.

Table 2.1 Electrical conductance of different soils (Salman *et al*., 2014)

Soil salinity	**Electrical conductivity (dSm^{-1})**	**Effects on plant**
Non saline	0-2	Negligible effect
Slightly saline	2-4	Yield of Salt sensitive crops affected
Moderately saline	4-8	Yield of many crops affected
Strongly saline	8-16	Tolerant crops affected
Very strongly saline	Above 16	Highly tolerant crops affected

2.3 Effect of salt on soil particles

The structure of soil is described by the arrangement of the soil particles, and the spaces between their macro- and micropores. High Salt concentration in soil affects its permeability. Soil particles get dispersed and clay particles swell up due to imbalanced infilteration rate of soil. Excess sodium ions in soil affects the binding forces of clay particles. This causes soil pores to become narrow and further quenching of dry soil aggregates causes reduction of wide pores and dispersed particles cause plugging of these pores (Abu-sharar *et al*., 1987). Hence water and solute flow gets arrested. The particles are captured in lower pores and thus within the soil structure water and air particles gets blocked (Roiston *et al*., 1984).

2.4 Effects of salinity on plant growth

Osmotic potential maintain transpiration and photosynthesis rate of plants and it gets reduced by soluble ions present in saline soil. Nutritional value of plants is also affected by direct exposure of chemicals. Aeration in saline soil gets hampered due to changes in physical structure of soil particles. It causes alterations in metabolic and biochemical process in plants. Various crops has different level of tolerance for salinity in soil, this can be defined by the threshold level of salinity. Plants root, shoot length and total dry weight also gets reduced significantly due to salt stress. Leaf number and leaf area also gets negatively affected due to harmful influence of salinity. Different plant species have significant threshold level of salinity tolerance, which can be seen in their responses in growth. Plant cell undergo changes around cell wall under salt stress as shown in figure 2.1. The ion channel is most affected by high sodium and chloride ions causing osmotic and ionic imbalance in plant cell.

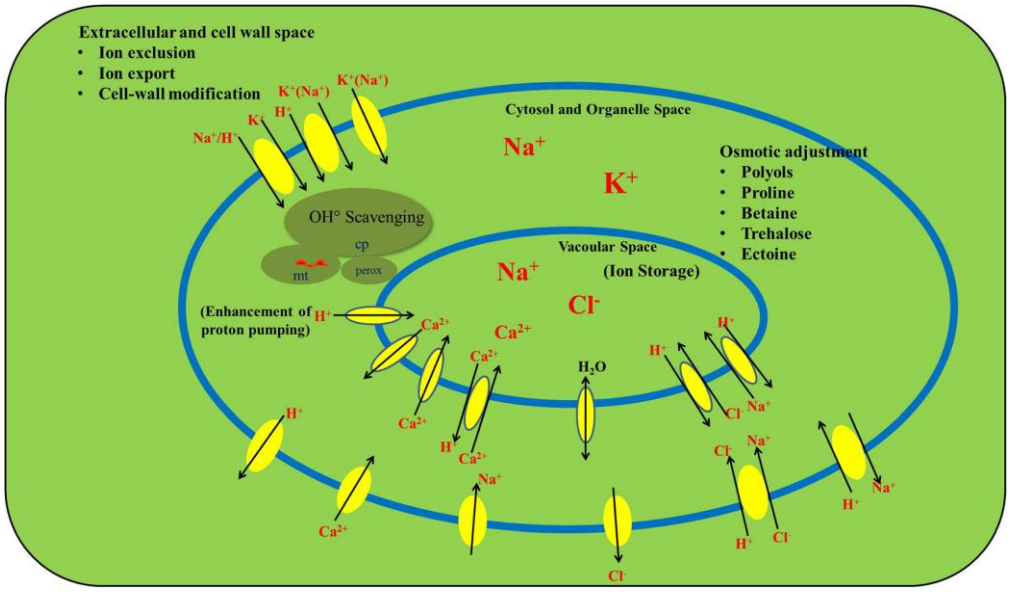


Figure 2.1 Changes in plant cell due to salt stress (Parihar *et al*., 2015)

Some visual symptoms that ocuur in crops as a consequence of salinity are stunted growth, marginal leaf necrosis and fruit quality degradation. Soil salinity decreases plant establishment in the soil, as wheat seedling emergence percentage may be reduced to 50 % in 8.8dSm^{-1} salinity level. Soil salinity also reduces primary and secondary tillers, number of leaves in main stem and number of spikelets per spike in wheat (Asadi and Khademi, 2013). The salinity threshold at which yield begin to decline have been reported to be 8.6dSm^{-1} for bread wheat. Each unit increases in soil salinity more than 10dSm^{-1} at 0 to 50cm of the soil layer, thereby causing wheat grain and straw yield reduction equal to 217Kgha^{-1} and

723Kgha^{-1}, respectively (Siadat and Sadat, 1998). Membrane stability of chickpea is related with the osmotic stress (Rawat *et al*., 2013).

2.4.1 Germination stage

Seed germination in many crops is the most basic and vital phase of plant growth. In maize and wheat, stage of germination is most affected by salinity in soil (Akbarimoghaddam *et al*., 2011). In many other plants altering in the enzyme activity, hormonal balance and osmotic balance reduces the utilization of seed reserves. Germination rate of *Zea mays* is reduced by 32% under 240mM NaCl concentration, as observed (Khodarahmpour *et al*., 2012). Bordi (2010) recorded a significant reduction in the proportion of germinating seeds in *Brassica napus* at 150mM and 200mM NaCl. Germination rate of many crops is negatively correlated with the increasing levels of salinity in soil (Kaveh *et al*., 2011).

2.4.2 Growth stage

Munns (2005) suggested that growth of plants is affected by two effects, osmotic effect and ion specific effect which gives the growth response in two phases:

Phase 1: It is the first phase in which growth of plant is affected by the higher concentration of salt accumulating in the soil solution. It decreases the growth of leaf, therefore reducing leaf area for photosynthesis and also to some extent of root growth (Munns, 1999).

Phase 2: In this phase, growth is affected by increasing salt inside the plant cell which is taken up by continous transport from soil to transpiring leaves. Toxic affects will causes death of leaves as high concentration of sodium and chloride ions accumulates in cell wall of plants and dehydrates the cell. Na^{2+} will interfere in the uptake of k^{2+} ions by plants and causes irregulation of stomatal openings on the other hand chloride ions has more adverse affects on plant growth (Tavakkoli *et al*., 2001) as it causes the chlorotic toxicity by disturbing the chlorophyll content. Salinity not only affects the seedling stage in plants as in *Oryza sativa*, this can be seen that plant height and leaf area gets reduced under salt stress conditions (Hasanuzzaman *et al*., 2009).

2.4.3 Photosynthesis

Decreased chlorophyll content and reduced water potential are the two main reasons affecting photosynthesis in plants. *Oryza sativa* when exposed to 100mM NaCl showed 30%, 45% and 36% reduction in chlorophyll a, chlorophyll b, and carotenoids contents respectively as compared to the control plant (Chutipaijit *et al*., 2011). Kalaji *et al*.,(2011) reported that in barley, photosystem 2 is adversely affected by salt stress, therefore reducing the chlorophyll content and function of oxygen evolving complex.

2.4.4 Salinity induced oxidative stress

Reactive oxygen species are highly reactive in nature produced under salinity stress as carbon fixation got inhibited due to stomatal closure (Ahmad *et al*., 2013). Cellular toxicity in crop plants is produced by damage to membranes of plant cell mediated by ROS (Mittova *et al*., 2004). In wheat seedlings, H_2O_2 levels increased under salt stress of EC 5-10 dSm^{-1} (Sairam *et al*., 2002), salinity in soil to a low level is directly propotional to antioxidant enzyme activities get increased but as salt stress increases, enzyme activity got reduced resulting in osmotic stress, in *Pisum sativum* (Ahmed *et al*., 2008). Some antioxidant enzymes are superoxide dismutase (SOD), ascorbate peroxidise (APX), peroxidase (POD), glutathionine reductase (GR), they scavange the reactive oxygen species and minimises the oxidative stress.

2.4.5 Nutrient imbalance

Impacts of salinity on nutrient uptake by plants causes nutritional disorders as there will be no sufficient amount of nutrients and minerals because of less availability in soil. Salt stress in soil causes pH of the soil to raise to a level which can cause many micronutrient deficiencies (Zhu *et al*., 2004). High levels of sodium chloride ions around the root regions reduces the nutrient assimilation, particularly K and Ca, leading to imbalanced ion channels (Keutgen and Pawelzik, 2009).

2.4.6 Yield of plants

Salt stress on plants contributes to a reduction in crop yields, which is a major effect of salt stress on agriculture. Linghe and Shannon (2002) reported that grain yield of rice is negatively correlated with salinity. Number of pods per plant, shoot and root weight also reduced. Greenway and Munns (1980), reported that if 200mM of NaCl salt stress is given to sugar beet, which comes in category of salt tolerant species, might have a reduction of only 20 % in dry weight. Whereas, in a moderately tolerant species such as cotton, same amount of salt concentration might cause reduction of its yields by 60%, and a sensitive species such as soybean might be dead.

2.4.7 Effects on crop plants

The adverse effects of salinity can be seen on crop plants. In field conditions, the wheat crop yield gets reduced in the presence of 100mM-175mM NaCl, showed a significant reduction in spikelets per spike, delayed spike emergence and reduced fertility, which results in poor grain yields (Munns, 1999).

Westcot and Ayers (1985), reported that yield of wheat is reduced by 50% at electrical conductivity of $13dSm^{-1}$, whereas there is difficulty in surviving of rice crop and yield gets reduced at $7dSm^{-1}$. Rice is the most important crop and a salt sensitive one, which limits its

availability in many regions. Maize is a staple crop and is three times more sensitive to salt stress than wheat (Ding *et al*., 2010). It has a threshold tolerance of salinity in between rice and wheat. Dry mass of maize gets reduced at 100mM NaCl concentration. Reduced yield of this crop leads to economic deprivations (Gadalla *et al*., 2007). Zoerb *et al*., (2004) stated there is loss of water due to osmotic stress in maize under 150mM NaCl stress. Barley comes in the category of the most salt tolerant cereal, that can tolerate up to 250mM NaCl beyond which the survival rates drop drastically. Sorghum is the main source of all the food components in the arid regions for millions of poor people. Protein content of sorghum is equal to wheat and maize. Salinity causes reduction in dry weight and yield of the crop. Under salt stress Na and Cl ions gets accumulated near roots of plants, in place of K and Ca ions (Farooq et al., 2015).

To overcome the problem of salinity stress, there must be an introduction of more salt tolerant crops like sorghum, as it is a moderately salt tolerant crop with the tolerance of 6-8dSm^{-1} (Greenway & Munns, 1980). It is not much affected by salinity and can produce higher biomass than other crops in same levels of salt stress. Rice or other crops cannot be grown in many salt affected areas where sorghum cultivation may become a solution.

According to Mass and Grattan (1999), annual crops are more sensitive to salinity in soil at their growth stage while they are much tolerant at germination stage but this process gets delayed with the salt stress. Reduction in Percentage of germinated seed in tomato and cotton (Licandro, 2002) and in chickpea (Kafi and Goldani, 2001) were observed. While wheat and sorghum are more sensitive during their vegetative growth phase. In rice, salinity increases sterility and shoot length is more affected as compared to the root length (Khatun *et al*., 1995).

The research of salinity tolerance in plants is very scarce and physiological processes that mediate tolerance are poorly understood. Therefore, there is a need to aim at accessing the impact of salt stress on many salt tolerant plants, their germplasms and developing seedlings. Some methods have been created to reduce the toxic impacts of elevated salinity on plant growth, introduction of salt tolerant crops by breeding methods (Glick *et al*., 2014).

2.5 Salt tolerance in plants

Salt tolerance in plants is their ability to grow and survive on a high concentration of soluble salts. Plants are categorised on the basis of salt tolerance as :

Halophytes - these are the salt tolerant plant species that can grow at a salt concentration of more than 200mM. Plants become adaptive to high salt with the help of useful enzymes (Parida and Das, 2005).

Glycophytes – These are salt sensitive plants that have stunted growth and even death at 100-200mM salt concentration.

There are differences between both the categories at a molecular level as halophytes have some stress tolerant genes. The primary mechanism which is present only in halophytes to overcome salt stress is by raising osmotic pressure in cytoplasm by production of some solutes, hence eliminating excess Na^+ ions from plant cells (Blumwald *et al*., 2000). The secondary mechanism for adapting salt stress conditions can be the endophytic connection between plant and bacteria that release some metabolites for plant growth. This mechanism can be seen both in halophytes as well as some tolerant glycophytes to overcome abiotic stress (Kloepper and Schroth, 1980). This continuing problem of soil salinity is determined by understanding crop protection against the stress and development of some biological methods to alleviate this issue.

2.6 Halophytic bacteria and their adaptive mechanisms

Halophytic bacteria resides in hypersaline environments and have adaptive mechanisms to grow at higher salt concentrations. They can be exploited for producing plant growth promoting metabolites under salt stress. Halotolerant bacteria can have some ameliorating effects on plants as shown in figure 2.2. They can promote plant growth by decreasing the salt uptake, and by increasing available phosphorus in soil.

Halophilic microbes inhabitats in hypersaline environments. Sambhar lake is the largest halo-alkaline lake in India. Sahay *et al*.,(2012) isolated 93 halophilic bacterial isolates from this lake, and screened them for plant growth promoting attributes. *Azospirillum*, *Bacillus*, *Halobaccilus*, *Halococcus*, *Pseudomonas* are the major species. Lakzian et al.,(2017), isolated halotolerant bacteria of *Virgibacillus* genera which can tolerate up to 320mM salt concentration and it can reduce the effect of salt stress on root growth of plants. A novel PGPB *Kushneria* spp. was isolated from saline region in china and identified using 16S rRNA gene sequencing. This species can tolerate up to 20% salt stress and showed P solublization at high concentration of salts (Zhu *et al*., 2011).

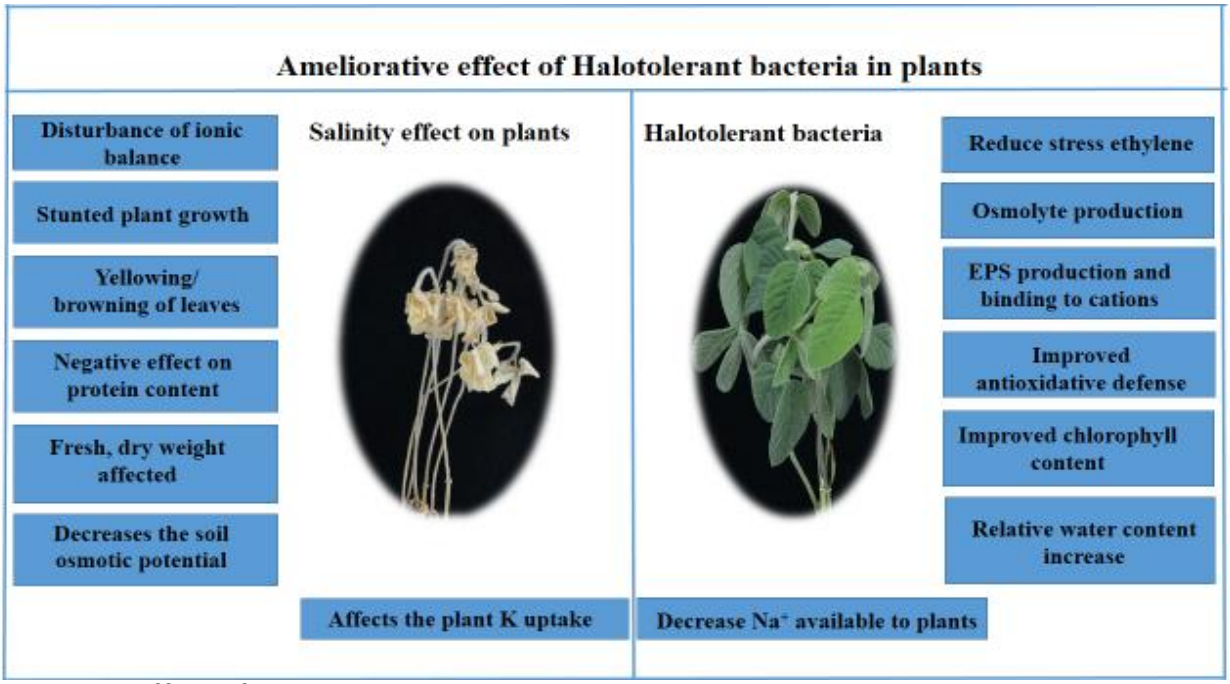


Figure 2.2 Effect of salt stress in plants and amelioration by halotolerant bacteria (Shin *et al*.,2016)

Tilak *et al*.(2005) reported a bacteria *Enterobacter cloaceae* Isolated from rhizosphere of *Aerya jayanica*, a commonly growing plant around Sambhar salt lake. This showed IAA production and P solublisation to a great extent. *Rhizobium*, *Azotobacter*, *Azospirillum*, *Bacillus* are the common bacteria residing in saline areas in india and has shown increased growth in plants (Anapadhyay *et al*., 2010).

2.7 Salt stress alleviation in plants by Plant growth promoting bacteria

Use of microorganisms is emerging these days to overcome salt stress in plants. Plant growth promoting bacteria are microorganisms that have natural potential to stimulate growth factors in plants. They can be free living or rhizospheric which colonize in the roots of plants, they are mainly known as plant growth promoting Rhizobacteria (PGPB). They enhance nutrients in soil by increasing the measure of accessible nitrogen and phosphorus and other plant supplements and a mixture of unique phytohormones that can demonstrate to improve different phases of plant development and furthermore eliminating soil borne pathogens by production of anti- microbial compounds.

In salt stressed plants, inoculation of plant with *Rhizhobium* in presence of *Azospirillum* increased flavonoids in plants. Therefore sometimes co-inoculation of different bacterial species have a more pronounced effect on plant growth (Upadhyaya *et al*., 2011). In canola seedlings, root length and dry weight increased by 40% under 150 mM salt stress, when inoculated with halotolerant bacteria *Micrococcus yunnanensis* and *Bacillus aryabhattai*

(Shin *et al*., 2016). In cotton plant, growth was improved with inoculation of *Pseudomonas putida* under salt stress (Yao *et al*., 2010). In this way, target microorganisms in soil were exploited for their useful traits to increase plant yield for agronomic utility.

2.7.1 Traits of PGPB

2.7.1.1 Phosphate solublization

Plants require soluble phosphorus in soil which they can take up for their normal growth (Feng *et al*., 2002). The phosphorus present in soil is mainly insoluble and not available to plants, therefore it is necessary to identify phosphate solublizing bacteria that can solublize both organic and inorganic forms of phosphorus and increase available P in soil (Fankem *et al*., 2006). Phosphate solublizing bacteria can use insoluble phosphate like rock phosphate, aluminium phosphate, tri-calcium phosphate in medium. Phosphate solubilizing microorganisms assume a significant job in enhancing phosphorus availability to the plants, permitting a sustainable utilization of phosphate manures. Many plant growth promoting bacteria (PSB) release low molecular weight organic acids by which insoluble phosphorus can be solublized and eventually taken up by plants. The hydroxyl or carboxyl group of organic acids chelate with the insoluble form of P and make it available to plants. PSB can form carbonic acids with evolution of CO_2 and can solublize calcium phosphate. On the other hand, organic forms of phosphorus can be hydrolised by the action of enzymes like phosphatase synthesised by bacteria. The mechanism of phosphate solublization by bacteria is shown in figure 2.3. Phosphatases are enzymes that hydrolyze phosphate groups from a wide variety of organic substrates, producing an alcohol and phosphoric acid. They are found in all cells and usually are classified as either acid phosphatases or alkaline phosphatases. These enzymes mineralise organic forms by dephosphorylation of phosphoester bonds (Achal *et al*., 2005).

The ability of solublizing insoluble form of phosphate can be seen in many heterotrophic and chemo-autotrophic organisms, and some fungi and actinomycetes. Bacterial species have higher capacity of solublizing phosphate by secreting organic acids, as compared to fungi (Alam *et al*., 2002). *Achromobacter, Agrobacterium, Bacillus, Pseudomonas* are few bacterial genera that have been accounted for solubilizing different amounts of phosphorus as they all vary in their efficiency for this activity. *Aspergillus* (Reddy *et al*., 2002) and some *Pseudomonas* are the main phosphate solublizers. *Bacillus circulans* isolated from rhizospheric soil of apple showed many plant growth promoting activities, made phosphorus available to plants, (Walia *et al*., 2014).

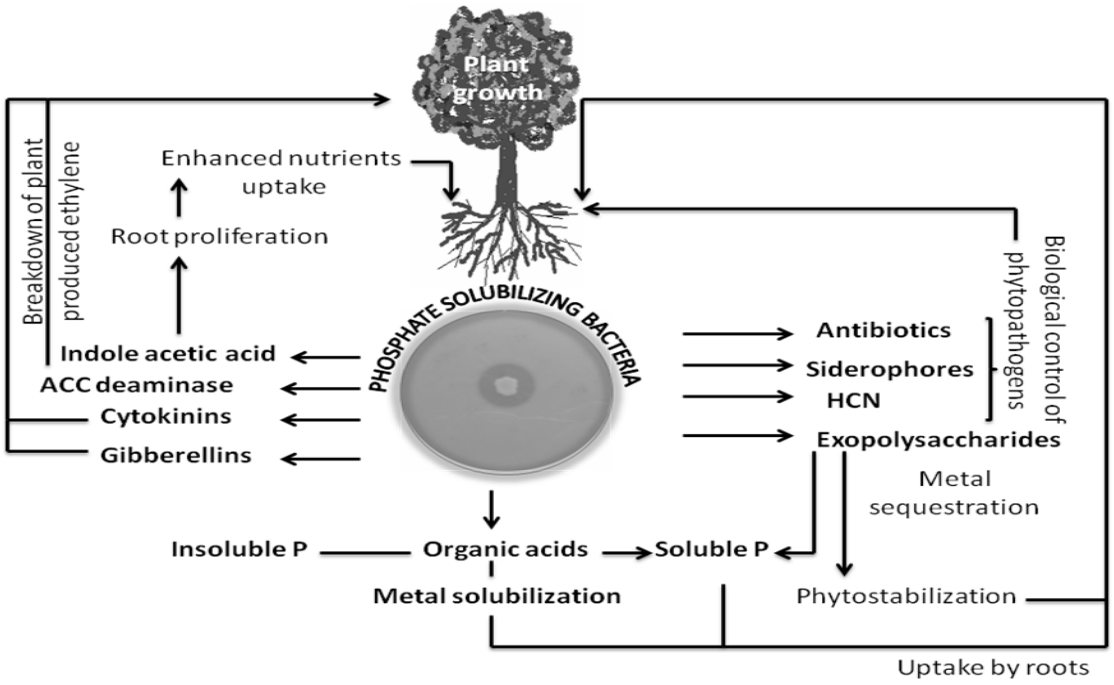


Figure 2.3 Mechanism of phosphate solublisation by bacteria (Zaidi *et al*., 2009).

It was reported that *Pseudomonas fluorescens* made phosphorus available in soil which enhances colonization of mycorhhiza in maize (Krey *et al*., 2013). *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean.

2.7.1.2 IAA production

Indole 3 acetic acid is the most important and common phytohormone which promotes plant growth. Mainly rhizospheric bacteria use tryptophan mediated metabolism to release auxins as secondary metabolites as compared to free living bacteria (Farah, 2004). Therefore IAA production largely affects root nodulation in plants, this will increase surface and length of roots, respiration rate, plant's metabolism that can ultimately help plants to take up large amount of available nutrients from soil (Okon *et al*., 1997). IAA production also improves fruitning and flowering in plants (Zhao, 2010). It was also reported that many IAA producing bacteria were *Pseudomonas* and *Azotobacter,* and some *bacillus* (Ashraf *et al*., 2013). These can also be called as root promoting bacteria.

2.7.1.3 Production of Siderophore

Siderophores are the iron chelating compounds produced by microorganisms. They form complex with the Fe^{+3} ions that are present in soil and this complex when enters in cytosol, ferric ions get reduced to ferrous ions which gets released from the complex and become the sole source of iron for plant growth. Siderophore producing bacteria will eliminate phytopathogens by limiting iron availability to them as most of the iron that is present in soil

is used by pathogens for their growth (Kohler *et at*., 2006). Fluorescent *pseudomonas* under salt stress showed Siderophore mediated pathway, reduces root colonization with pathogens and promotes plant growth (Decheng *et al*., 2005). Siderophore producing microbes increases plant biomass in cucumber. Soil fertility also increases (Ali and Vidhale, 2013).

2.7.1.4 HCN production

Indirect mechanisms of plant growth promotion are by production of some biocontrol agents (Glick, 2012). Bacteria producing hydrogen cyanide can be used as biological control for pathogens as it is a powerful antimicrobial compound known (Haas *et al*, 2005). Particularly HCN producing bacteria helps in plant growth by the mode of action of disease suppression. It eliminates unwanted growth of fungus that degrades many crops.

Chapter- 3

MATERIALS AND METHODS

3.1 Collection of samples

Samples were collected from sediments near the saline brines of Sambhar salt lake, Sambhar, Rajasthan, India. Total six samples were taken from different portions near these brines, carried aseptically in test tubes. After the collection they were stored at 4°C until further processing to isolate halophilic bacteria.

3.2 Determination of pH and electrical conductance of sample

pH of the sample was determined using Eutech instruments pH meter.

3.2.1 Procedure

- Sample in water suspension solution (1:2.5) was made.
- Stirred with a glass rod for 2-3 min.
- Kept it at a shaker with 130rpm for 2hrs., in room temp, allowed to settle.
- pH was recorded when reading got stabilized.

3.2.2 Elertrical conductance

EC of soil sample was determined using SPECTRALAB instruments Pvt. Ltd. Conductivity-TDS meter.

Procedure

- Suspension mixture was made same as above.
- The electrode was dipped in the suspension solution and recorded the reading displayed for EC.

3.3 Enrichment of sample

Sample was enriched in 100ml complex halophilic medium supplemented with 5% NaCl for 24hrs. at 37°C on shaking conditions.

3.4 Isolation of halophilic bacteria

Table 3.1 Composition of complex halophilic medium (Sehgal and Gibbons, 1960).

Components	Concentration (g/l)
NaCl	100
KCl	2
MgSO4.7H2O	2
Dextrose	20
Yeast extract	10
Peptone	7.5
MnCl2.7H2O	0.36mg/l
FeSO4.6H2O	1.6mg/l
Agar agar	20

Procedure

- All media composition were added and autoclaved at 15psi for 15min.
- After autoclaving, the media was poured in sterilezed petri plates in laminar air flow
- Solidified plates were used after 24hrs (to check any contamination).
- From the enriched sample, serial dilutions were made with normal saline. 1 ml of sample was added in 9ml of normal saline to make $1x10^{-1}$ dilution.
- Up to $1x10^{-7}$ dilutions were made. 100µl of each dilution was spread on complex halophilic agar medium plates.
- Incubated the plates at 37°C for 24hrs.
- Isolated colonies were streaked on sterile medium plates for further analysis.

3.5 Estimation for salt tolerance of bacterial isolates

Salt tolerance of bacterial isolates was done by estimating colony forming units on media agar plates.

Procedure

- Purified isolated colonies were inoculated in peptone water overnight.
- After incubation, 1ml of culture was transferred to fresh peptone broth.
- Incubated at 37°C for 24hrs. on shaker.
- Prepared the broth supplemented with different NaCl concentrations in test tubes.
- Transferred 1ml of the broth culture to each test tube, incubated it at 37°C.
- Serial dilutions was made up to 10^{-5} and 100µl was spreaded on the halophilic media agar plates.
- After incubation for 24hrs., colony forming units was measured and log cfu/ml was calculated

3.6 Plant growth promoting activities by halotolerant bacteria

3.6.1 Production of Indole 3 acetic acid (Brick et al., 1991)

All the selected halotolerant bacterial isolates were quantified for IAA production.

Reagents

- Yeast malt dextrose broth
- Salkowski's reagent – one ml of 0.5M $FeCl_3$ added in 50ml of 35% perchloric acid. Stored in amber coloured bottle.

Table 3.2 Composition of yeast malt dextrose broth

Components	Concentration (g/l)
Peptone	5
Yeast extract	3
Malt extract	3
Dextrose	10
Final pH	6.2
L-tryptophan	100µg/ml

Procedure

- Yeast Malt Dextrose broth containing 100µg/ml tryptophan was prepared and autoclaved at 15psi for 15min.
- Broth was then inoculated with 1ml of previously enriched bacterial cultures and a control without any inoculum was maintained.
- Incubated at 37°C for 5 days on a shaker with 130rpm.
- After incubation, culture was centrifuged at 8000rpm for 10min.
- One ml of supernatant was taken in a test tube, added 2ml of freshly prepared salkowski's reagent and kept in dark for 30min.
- Absorbance of pink color produced after incubation was taken at 540nm using Spectrophometer.
- Uninoculated sample was taken as blank.
- Standard curve was plotted by preparing stock solution of 1 mg/ml IAA.

Table 3.3 standard solution of IAA

Concentration (µg/l)	Amount of stock added (ml)	Amount of media added (ml)
0	0	10
5	0.05	9.5
10	0.1	9.9
20	0.2	9.8
50	0.5	9.5
100	1	9

3.6.2 Qualitative estimation of phosphate solublization by bacteria

Pikovskaya's agar plates containing tri-calcium phosphate was used to check phosphate solublization of bacterial isolates (Pikovskaya, 1948).

Table 3.4 Composition for Pikovskaya agar medium

Components	**g/l**
Dextrose	10.0
Yeast extract	0.50
Tri-calcium phosphate	5.00
Potassium chloride	0.20
Ammonium sulphate	0.5
Magnisum sulphate	0.10
Ferrous sulphate	0.0001
Distilled water (vol. make up)	1000ml
pH	7.0
Agar	15.00

Procedure

- Pikovskaya agar medium plates were made in sterile conditions**.**
- Halotolerant bacterial isolates were spot inoculated on Pikovskaya agar plates.
- Incubated at 37°C for 2-3 days, plates were checked at regular intervals for bacterial growth
- Phosphate solubilization was visible by halo zone around bacterial colonies..
- Phosphate solublization index was calculated using following formula as suggested by Premono *et al*., (1996).

$$\text{Solublization index (SI)} = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

The halo zone of phosphate solubilization around growth was recorded (in mm) after every 24 hours of incubation.

3.6.3 Ammonia production

Ammonia production by bacterial isolates was estimated by nesselerization reaction

Reagents

- Peptone broth – It was prepared by adding 10g of peptic digest and 5g of NaCl in 1000ml distilled water.
- Nessler's reagent

Procedure

- Inoculated selected bacteria isolates to sterile peptone broth.
- Incubated at 37°C for 24- 48hrs.
- After incubation, centrifuged the culture at 8000rpm for 10min.
- Equal amount of Nessler's reagent to the supernatant (1.5 ml) was added.

- Observed the colour change as indicator of ammonia production and took O.D at 425nm (Demutskaya & Kalinichenko, 2010).
- Concentration of ammonia produced by bacterial isolates was determined by a standard curve of ammonium sulphate.

3.6.4 HCN Production

Determination of HCN production was done according to Bakker and Schippers, 1987.

Reagents

- 2% (w/v) of sodium carbonate in 0.5% (w/v) of picric acid.

Procedure

- Nutrient agar plates amended with 4.4g/l glycine, were prepared.
- Inoculated bacteria isolates on nutrient agar plates with an inoculating loop.
- Whatmann filter paper strips were dipped in 2 % w/v sodium carbonate in 0.5% (w/v) picric acid solution and were placed inside the lid of the petriplates.
- Plates were incubated at 37°C for 4 days.
- Color change of the filter paper from yellow to brownish indicate HCN production.

3.6.5 Siderophore production

Siderophores directly or indirectly involved in plant growth, their production by the isolates can be estimated on CAS agar medium (Schwyn and Neilands, 1987).

Reagents

- CAS dye – Dissolve 60.5mg of CAS reagent in 50ml distilled water. 1mM of $FeCl_3.6H_2O$ solution was prepared in 10ml of 10mM HCl solution. Dark reddish color appeared. In a separate beaker, 72.9mg of CTAB was dissolved in 40ml distilled water. With continuous stirring, both the solutions were mixed, which results in dark blue color. Autoclaved the CAS dye separately.
- CAS agar plates – 100 ml of CAS dye was added in a 300 ml autoclaved nutrient agar medium and before pouring to plates, pH was checked with pH strips and maintained at 7 using autoclaved NaOH or HCl accordingly.

Procedure

- Bacterial colony was inoculated on CAS agar plates with an inoculating loop.
- Incubated the plates at 37°C for 3-4 days.
- Change of blue color of the medium to orange yellow color around the bacterial colony indicated siderophore production.

3.7 Quantitative estimation of PGP attributes at different salt concentrations by bacterial isolates.

3.7.1 IAA production

Procedure

- Yeast malt dextrose broth was supplemented with NaCl concentrations (5%- 20%).
- Bacterial isolate was inoculated in each of the broth.
- Earlier explained procedure was followed, and concentration of IAA produced was estimated by each isolate at different level of NaCl.

3.7.2 Quantification of phosphate solublisation

Chlorostannous reduced molybdo phosphoric acid blue method by Jackson, 1973**.**

Reagents

- Chloromolybdic acid – In 400ml distilled water, 15g of ammonium molybdate was dissolved and filtered in a beaker, slowly added 400ml of 10N HCl with continuous stirring. Total volume was made up to 1000ml with distilled water and this was then stored in amber glass bottle.
- Chlorostannous acid – Stock solution was prepared by dissolving 10g of stannous chloride in 25ml of conc. HCl and stored in airtight glass bottle.

Working solution – 1ml of the stock was added to 132ml of distilled water. This solution was freshly prepared, each time.

Procedure

One ml of culture was transferred to 100ml PKV broth, incubated for 5-7 days at 37°C on shaker. After incubation the culture was centrifuged at 10000rpm for 15 min. 500µl of supernatant was taken in a flask, added 10ml of chloromolybdic acid to it. After addition of 1ml of chlorostannous acid, the final volume was made up to 50 ml with distilled water. Blue color formed and the intensity of the color was noted by taking absorbance at 660nm. The concentration of Phosphate solublized in each sample was estimated by obtaining regression equation from standard curve of KH_2PO_4 at 600nm. There was also a reduction in pH of the culture broth which can also be noted. Quantitative estimation of phosphate solublisation was also done at 5% - 20% salt concentrations following the same procedure.

3.7.3 Estimating Siderophore production

Quantitative estimation of Siderophore in broth (Payne, 1994)

Procedure

- Nutrient broth was inoculated with bacterial isolate and incubated at 37°C for 3 days at 120rpm.
- After incubation, culture broth was centrifuged at 10000rpm for 15 minutes.

- To 1.5ml of supernatant, 1.5ml of CAS dye solution was added, kept for 30 minutes undisturbed.
- Loss of blue color was observed in case of siderophore production when compared to control. Absorbance was taken at 630nm.
- Concentration of siderophores produced by bacteria was calculated as:

$$\% \text{ Siderophore units} = \frac{Ar - As}{Ar} \times 100$$

Where,

Ar= Absorbance of reference at 630nm (CAS uninoculated broth & CAS dye)

As = Absorbance of the sample at 630nm

Siderophore production at different salt concentration was observed by amending the medium with 5%, 10%, 15%, 20%.

3.7.4 Ammonia production at different salt concentrations

Procedure

Quantitative estimation of ammonia was done at 0%-20% salt concentrations using the same procedure as explained earlier.

3.8 Biochemical characterization of selected bacterial isolates

3.8.1 Gram staining

- A clean glass slide, wiped with ethanol was taken
- Smear of bacterial culture was made, air dried followed by heat fixing.
- Flooded with crystal violet stain for 1 minute, washed with water using wash bottle.
- Flooded with Gram's iodine for 1 minute, again washed with water.
- Slide was washed off using decoloriser from the staining kit or 95% ethyl acetate for 5 seconds so that no violet color would be seen on the smear.
- Again washed with water and counter stained with safranin for 30 seconds.
- Washed with water dropwise and was air dried or with absorbent paper.
- Slides were examined under light microscope.
- Colonies appearing purple violet colored were gram positive bacteria and if orange red coloured, were gram negative.

3.8.2 Catalase test

- Took a clean glass slide.
- Bacterial colony was placed on slide with the inolulating loop.
- Added 1-2 drops of hydrogen peroxide.

- Bubbles were formed indicating production of oxygen.
- Later appearance of bubbles was considered to be negative.

3.8.3 Oxidase test

- Took a glass slide and isolated bacterial colony was placed on it.
- Placed oxidase discs (HiMedia, India), on it.
- Observed the color change within 10 seconds.
- Deep purple color gives positive results**.**

3.8.4 Nitrate reduction test

- Nitrate agar placed were used, bacteria was inoculated on the plate using inoculating loop.
- Nitrate discs were used, manufactured by HiMedia, India.
- Disc (part A) was placed on bacterial colony and added few drops of rehydrating fluid (part B).
- Observed the color change, dark red color was formed indicated the positive result.

3.9 Molecular characterization

3.9.1 Genomic DNA isolation

Procedure

- Bacterial culture was inoculated in 20ml nutrient broth for 16-24 hrs. on a shaker at 37°C.
- 2ml of culture was taken in a microfuge tube and Cells were centrifuged at 10000rpm for 10 min.
- Supernatant was discarded.
- Pellet was resuspended in 0.8ml saline EDTA buffer.
- Lysozyme solution was freshly prepared and 50µl was added to the tube, incubated at 37°C for 20-30 min.
- 0.2 ml of 10% SDSwas added, mixed by inverting the tube, this was then incubated at 60°C in a water bath for 15 min.
- Taken out from water bath, equal volume of phenol chloroform isoamyl alcohol (25:24:1) was added , mixed by inversion and centrifuged at 12000rpm for 10min.
- Transferred the upper aquous phase in another sterile microfuge tube, equal volume of chilled isopropanol was added with gently mixing to precipitate DNA.

3.9.2 Gel electrophoresis (qualitative estimation)

- 1 % of agarose gel was prepared in 0.5X TBE buffer, heated to have a clear solution.
- Allowed to cool at room temperature, ethidium bromide was added to it.
- Poured on gel casting tray having combs to make wells for loading of DNA sample.
- Gel was allowed to solidify for 30 min.
- DNA samples(6µl) were loaded with 4µl of 6X loading dye.
- Nucleic acid was electrophorised at 70V for 1hr.
- DNA bands were visualised under UV transilluminator.

3.9.3 Quantitative estimation using Thermo scientific, Wilmington, DE Nano drop 1000 spectrophotometer.

Concentration of DNA was quantified on nanodrop and calculated by A260/A280 ratio, which should be in range of 1.8- 2.0.

3.9.4 PCR amplification of 16S rDNA

Requirements- DNA samples, Deoxynucleotides triphosphates (dNTP's), 10X buffer, DNA (Taq) polymerase, $MgCl_2$ (1.5mM), forward and reverse primer, sterile MQ water, PCR tips, micropipetts .

Forward primer: 5'-AGA GTT TGA TCC TGG CTC AG-3'

Revrse primer: 5'-ACG GGC GGT GTG TTC-3'

Table 3.5 Composition of PCR reaction

Component	Concentration	Volume for 1 reaction (µl)
MQ water		12.2
PCR buffer	10X	2
DNA template	10ng	1
dNTPs		1.5
$MgCl_2$	1.5mM	1
Forward primer	0.2mM	1
Reverse primer	0.2mM	1
Taq polymerase	2.5U/µl	0.3

Table 3.6 Reaction conditions for PCR

Cycle	Temperature	Duration	
Initial Denaturation	92°C	2 min.	
Final Denaturation	92°C	1 min.	35 cycles
Annealing	50°C	30 sec.	
Initial extension	72°C	2 min.	
Final extension	72°C	7 min.	

Procedure

- Amplification of 20µl reaction mixture was done with 35 cycles of amplification in GenAmp 2700 thermocycler**.**
- Control reaction mixture was also made without DNA template.
- Aliquots of 6µl amplified product was made and were electrophoresed in 1% agarose gel.
- Bands were visualised on UV transilluminator.

3.9.5 Purification of PCR product

Purification was done using QIAquick gel extraction kit (QiageInc., USA)

Procedure

- PCR products were purified by gel excision purification.
- Protocol was followed as per manufacturer's instructions.
- Purified PCR product was suspended in 30µl MQ water.

3.10 Nursery experiments

Selected bacterial isolates were investigated for their plant growth promoting attributes under salt stress, HB9 and HB19 were halophilic bacterial isolates which showed IAA production, phosphate solublization, ammonia and siderophore production. Experiment was conducted on sorghum and seeds were inoculated with individual as well as bacterial consortium of both the isolates.

3.10.1 Seed treatment

3.10.1.1 Seed inoculation

Reagent- For slurry preparation**,** 10% sugar solution and 40% gum acacia in sugar solution.

Procedure

- Bacterial culture was inoculated in nutrient broth.
- Incubated on a shaker at 37°C for 24hrs.
- Centrifuged at 8000rpm for 10min, supernatant was discarded
- Pellet was resuspended in sterile distilled water.
- Repeated the last step.
- Slurry was prepared freshly by dissolving 10g of sugar in 100ml distilled water.
- Sugar solution was heated on hot plate and 40g of gum acacia was added to it.
- Mixed with a glass rod, cooled at room temperature.
- Once the slurry got prepared different bacterial inoculums were added seperately and mixed well.

3.10.1.2 Disinfection of seeds

- Seeds of sorghum were taken in a beaker, washed with disinfectant.
- Ethanol was added and covered beaker with aluminium foil.
- Decanted the ethanol and washed again with distilled water.
- Added bleach solution or 3% sodium hypochlorite till seeds got covered, rest it for 5min.
- Decanted the bleach solution, washed with distilled water.
- Rinse 2-3 times with sterile distilled water.
- After last washing, seeds were soaked in sterile distilled water and covered with foil, for 24hrs.

3.10.1.3 Coating of seeds with inoculum

Washed seeds were taken and dried, they were mixed with the slurry inoculum mixture and coated well. Total three slurry mixtures were made: a) with HB9, b) with HB19, c) with HB9+HB19. Uniformly coated seeds was done and were dried in dark conditions overnight and then sown in pots.

3.10.2 Experimental preparation for cultivation of plants

Nursery trial was conducted at the field area of core, Thapar Institute Of Engineering and Technology, Patiala on sorghum seeds with salt stress of 75mM and without salt was done in plastic bags. Seeds were sown in first week of April. Salt stress was maintained by irrigating with 75mM NaCl concentration once a week. Three bags of each treatment were set up and five seeds were sown in each bag. Treatments performed as follows**:**

- CONTROL without salt stress
- CONTROL with salt stress
- HB9 without salt stress
- HB9 with salt stress
- HB19 without salt stress
- HB19 with salt stress
- HB9+HB19 without salt stress
- HB9+HB19 with salt stress

3.10.3 Plant analysis after harvesting

No chemicals or natural fertilizers were added during the treatment. The crop was harvested in first week of July, for measuring growth parameters. Three plants per pot were randomly selected for measuring Shoot length, Root length, Fresh and Dry Biomass. These plants were dried at 65°C for 24 hrs in an oven, and checked for dry shoot and root weight and P content

in shoots. Soil samples from pots were also analyzed for pH and electrical conductance, elemental analysis- total P, C, N content, enzyme activities- acid and alkaline phosphates activity.

3.11 Plant and soil analysis

3.11.1 Determination of Soil pH

Determination of pH and electrical conductance of the sample was measured using 1:2 or 1:5 soil water suspension solution. Procedure was followed as discussed above for sample analysis.

3.11.2 Determination of available phosphorus

Available phosphorus in soil sample is estimated as Olsen et al., 1954.

Reagents for the estimation of available phosphorus

- 0.5M NaHCO3 extracting solution – 42g of sodium bicarbonate was dissolved to distilled water and final volume was made up to 1L. pH of this solution was adjusted using 1M NaOH to 8.5.
- Reagent A – Added 12g ammonium molybdate in 250ml of water. Seperately added 0.2908g of antimony potassium tartarate in 100ml distilled water. Both of the above solutions were added to 1000ml H_2SO_4 of 2.5M conc. Final volume was made up to 2Lwith distilled water.
- Reagent B – 200ml of reagent A, 1.058g of ascorbic acid was dissolved freashly for the use.
- Sulphuric acid - 1L of 2.5M sulphuric acid was made
- Stock standard P solution (50ppm) – 0.2917g KH_2PO_4 dissolved in distilled water to make 1L volume.
- Working standard P solution(1ppm) – 20ml of stock solution was diluted with distilled water to 1liter.

Procedure

- In 50ml of extracting solution, 2.5g of soil was added.
- Kept it on shaking conditions for 30 minutes and then it was separated through paper no. 42 whatman channel.
- Aliquot of 10ml filterate was moved to a 100ml container.
- 1ml of 2.5M H_2SO_4 and 15.5ml of distilled water was added to the filterate.
- 8ml of reagent B was taken and mixed with 15.5 ml distilled water and finally added to the above mixture.

- Solution becomes colored within 10min. and intensity of color was measured against blank (without soil mixture) at 880nm.

  Standard solution was prepared using 0, 2, 5, 10, 15, 20 ml of 1 ppm concentration of working solution, to this 10ml of extracting solution, 2.5ml H_2SO_4, 8ml of reagent B was added and final volume was made to 50ml. the total P concentration in these solution becomes 0.04 ppm, 0.1 ppm, 0.2 ppm, 0.3 ppm and 0.4 ppm respectively. After 10 min., OD readings were taken at 882nm and standard curve was plotted.

3.11.3 Total phosphorus estimation (Kitson and Melon, 1944)

Reagents

- Vanadomolybdate solution – two solutions were separately prepared and then mixed.
- Solution A- 25g of ammonium molybdate was dissolved in 300ml distilled water.
- Solution B - 300ml of distilled boiled water, 1.25g of ammonium vandate was added to it, allowed to cool down. 250ml of conc. HNO_3 was added and again cooled following addition of previously prepared 300ml solution A. Final volume was made to 1000ml with distilled water.

  Standard stock solution of P (50mg/ml) - 0.2195g of dried KH_2PO_4 dissolved in distilled water with thorough mixing. 25ml of 7N H_2SO_4 was added following distilled water to make up to a final volume of 1L.

Sample preparation for elemental analysis

Method of Wet oxidation of sample using di acid (HNO_3 – $HClO_4$) was performed for releasing mineral elements from soil sample for their quantification.

Procedure for elemental analysis

- In a digestion tube, 1g of air dried soil sample was weighted and 10ml conc. HNO_3 digest was added and heated for 1hr at 145°C using electric heater in a acid proof digestion chamber having fume exhaust.
- Allowed to cool down.
- Added 5ml $HClO_4$ and 10ml of conc. HNO_3, heated at 100°C, after an hour temp. raised up to 200°C.
- Digestion was continued until the contents become colorless and only white fumes were generated.
- Acid contents were reduced and white matter left in the digestion tube.
- White matter was allowed to cool and then added 50% diluted HCl.
- Filtered out using whatmann filter paper no. 42.

- Filterate was washed for 2-3 times and finally diluted to 50ml, both with 50% HCl.

Procedure for estimation of total phosphorus

- Equal volumes (10ml each) of vanadomolybdate solution and acid digests of soil sample was mixed and diluted to 50ml with distilled water.
- After 10 min. intensity of developed color was measured at 420nm.
- Standard of P was prepared using 0, 1, 2, 3, 4, 5 ml of stock solution mixed with vandamolybdate solution and water.

Calculations

$$P\ (mg/kg) = \frac{\text{Vol make up after digestion}}{\text{vol of digest used to develop color (ml)}} \times 50 \times P\ (mg/l)\ \text{weight of sample (g)}$$

3.11.4 Organic carbon estimation

Organic carbon in soil sample was estimated using a method given by Walkley and Black, 1934.

Reagents

- 1N potassium dichromate – 49.04g of potassium dichromate was dissolved in distilled water to a volume of 1L.
- 0.5N ferrous ammonium sulphate – 198g of ferrous ammonium sulphate dissolved in distilled water and volume was made up to 1L.
- Di phenyl amine indicator(DPA) - 0.5g of DPA was added to a mixture of 200ml of distilled water and 100ml of H_2SO_4.
- Conc. H_2SO_4
- 85% orthophosphoric acid
- Sodium fluoride

Procedure

- In a 500ml flask, 1g of soil sample was added with 10ml of 1N potassium dichromate and mixed properly.
- After this, 20 ml of conc. H_2SO_4 was added and kept undisturbed for 30 min., after which 200ml of distilled water was added.
- 1ml of DPA solution was added.
- This was then titrated with 0.5N ferrous ammonium sulphate which was freashly prepared, till blue color changes to green.
- A blank was run according to the above steps without soil sample.

Calculation

$$\text{Organic carbon (\%)} = \frac{10\ (\text{B} - \text{T}) \times 0.003 \times 100}{\text{B} \times \text{weight of soil (g)}}$$

3.11.5 Estimation of total nitrogen (Piper, 1960)

Reagents

- Anhydrous sodium sulfate
- 0.5gm Copper sulfate (catalyst)
- Concentrated sulphuric acid
- 0.02N Sulphuric acid: 0.272ml of concentrated sulphuric acid in 500ml.
- Boric acid solution: 20g of boric acid dissolved in 1000ml distilled water.
- Mixed indicator solution: 0.066g of methyl red and 0.099g of bromo cresol green was dissolved 100ml distilled water.
- 0.25N standard sodium hydroxide solution: 13.201ml of NaOH solution in 200ml distilled water and final volume made upto 1litre.
- Digestion mixture: 10g sodium sulfate, 0.5gm copper sulfate, 30ml concentrated H_2SO_4.

Procedure

- 2g of soil sample was taken in kjeldahl flask, 0.5gm copper sulfate was added in 30 ml H_2SO_4 and after digestion 50ml water was added.
- Flask was kept in digestion chamber at 100 C˚ for 2-3 hours.
- Color changed was monitored from dark brown to greenish white, contents were cooled and 50ml distilled water was added.
- Boric acid was taken in a beaker into which tubes were dipped.
- Glass beads were added in distillation flask containing digested sample.
- The volume of liquid in the distillation flask should not exceed half the capacity of flask to prevent the frothing.
- Sodium hydroxide was added to the distillation flask through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
- Condenser should be arranged to dip the tube in the beaker containing 50 ml boric acid.
- Distillate one third of the total volume of the solution in the flask.Assembly was cooled and dismantled.
- Distillate was collected through receiver tube containing 15ml boric acid and 2-3

drops of mixed indicator was added.

- The distillate containing ammonia was titrated against sulphuric acid till grass green color changes to steel grey.

Calculations

Nitrogen (% by mass) = $\frac{1.4 \times V \times N}{W}$

Where,

- V is the volume in ml of standard sulphuric acid used in titration.
- N is the normality of standard sulphuric acid.
- W is the mass in g of sample taken for the test**.**

3.11.6 Enzyme activity of soil

Acid and alkaline phosphatase activity

Reagents

Table 3.7 Composition of 5X modified universal buffer (Skujins et al, 1962)

Components	**g/l**
Tris amino methane	12.10
Boric acid	6.28
Malice acid	11.60
Citric acid	14
NaOH	488ml
Volume was made up with distilled water	

pH of the buffer was set to 5 using 0.5N HCl for acid phosphatise assay and for alkaline phosphates activity assay , pH of this buffer was adjusted to 9 with 0.5N NaOH.

- P-nitrophenyl phosphate solution - 4.268gm disodium p- nitrophenyl phosphate hexahydrate salt was dissolved in 100ml of appropriate pH adjusted, diluted MUB (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase.
- P-nitrophenol (1mg/ml)- This solution was also prepared in pH 5 MUB for acid phosphatase assay, and pH 9 MUB for alkaline phosphatase assay.
- 0.5N NaOH

Procedure

- 1g of dried soil sample was taken in a flask
- 4.0ml of the diluted MUB (pH 5.5 for acid phosphatase and pH 9.0 for alkaline phosphatase) was added to it.

- 1 ml 0.115 M p- nitrophenyl phosphate solution was added to the flask, mixed the contents.
- The flasks were incubated at 37°C for 1 hour in dark .
- After incubation, 4 ml of 0.5M NaOH was added to stop the reaction.
- Mixture was swirled and filtered through whatman filter paper no.2.
- Transferred the filtrate to glass cuvette and measured the yellow color at 410 nm.
- Phosphatase activity was indicated as the amount of p-nitrophenol released in the filtrate from the p-nitrophenyl phosphate substrate per gram of soil. The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standard containing 0, 10, 20, 30, 40, 50 µg of p-nitrophenol.
- To perform control, followed the procedure described for the assay but made the addition of 1 ml of p-nitrophenyl phosphate after the addition of 0.5 N NaOH (i.e. immediately before filtration).

Chapter- 4

RESULTS

4.1 Collection of sample

Sample was collected from Sambhar salt lake, Rajasthan (Figure 4.1) for the isolation of halophilic bacteria, to exploit their plant growth promoting attributes.

Figure 4.1 View of Sambhar salt lake, Rajasthan

4.2 Determination of pH and electrical conductance of the sample

The sample was slightly alkaline with pH of 8.9 and electrical conductance was observed to be 3.6dSm^{-1} which shows that the sample collected from Sambhar salt lake was highly saline.

4.3 Isolation of salt tolerant bacteria

Halophilic bacteria were isolated on complex halophilic medium containing 10% NaCl using serial dilution method. Isolated colonies can be seen in Figure 4.2. Individual bacterial colonies obtained were streaked on fresh medium plates in order to get pure isolates.

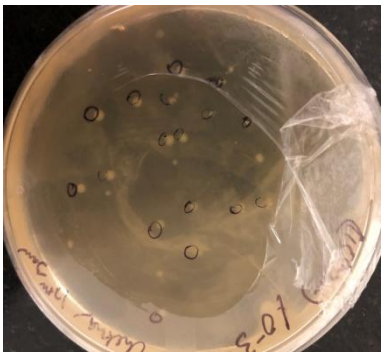


Figure 4.2 Bacterial colonies on complex halophilic medium

A total of 22 bacterial colonies were isolated and streaked on agar plates as shown in Figure4.3. After studying their morphological characteristics such as colony color, size, shape, Gram staining reaction, they were streaked on a fresh agar plates as patches for further screening for their salt tolerance. They were named as HB1 to HB22.

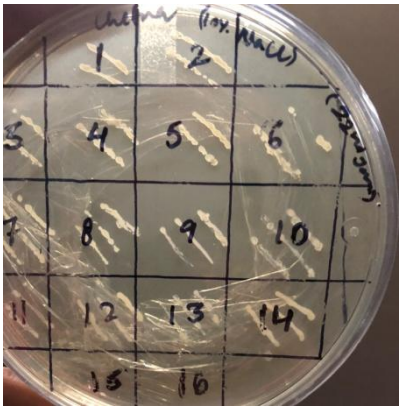


Figure 4.3 Bacterial isolates on complex halophilic medium amended with 10% NaCl

4.4 Salt tolerance of bacterial isolates

Screening of salt tolerance of bacterial isolates was done at different salt concentrations- 5%, 10%, 15% and 20%. Total of 10 isolates were selected based upon their initial tolerance to 10% NaCl in the medium and their colony forming units at these concentrations was determined.

Table 4.1 CFU/ml of 10 bacterial isolates at different salt concentrations.

Bacterial isolate	**log Cfu/ml value of each isolate at different salt concentrations**				
	Without NaCl	**5% NaCl**	**10% NaCl**	**15% NaCl**	**20% NaCl**
HB1	10.72	10.03	9.34	7.6	-
HB2	10.84	10.18	9.42	8.1	7.09
HB4	11.63	9.74	9.31	8.4	8.01
HB5	11.30	10.16	9.11	8	7.02
HB6	10.93	10.02	9.03	7.8	7.06
HB9	11.57	10.94	9.47	8.4	8.01
HB11	11.32	10.87	9.60	8.1	7.32
HB12	10.82	9.86	8.2	-	-
HB14	10.21	9.42	8.01	-	-
HB19	11.65	10.62	9.25	8.3	7.40

In this experiment, the most halotolerant bacteria were able to grow at 20% salt concentration. Table 4.1 shows that with increasing concentration of NaCl, growth of bacteria decreases. Finally seven bacterial isolates that showed high salt tolerance were further selected for testing their various plant growth promoting activities.

4.5 Plant growth promoting activities

4.5.1 IAA production

In the presence of L-tryptophan, there is formation of a complex Tris-(indole-3-aceto iron) which gives pink color in the broth. Bacteria use tryptophan mediated pathway for production of IAA. It was quantified after 5 days of incubation using yeast malt dextrose broth with 100 µg/ml of L-tryptophan. Pink color was observed as in Figure 4.4. Absorbance was taken at 530 nm. Out of seven isolates, only two bacterial isolates showed IAA production. Those were screened for phosphate solublization, and estimated for their activity at different salt concentrations.

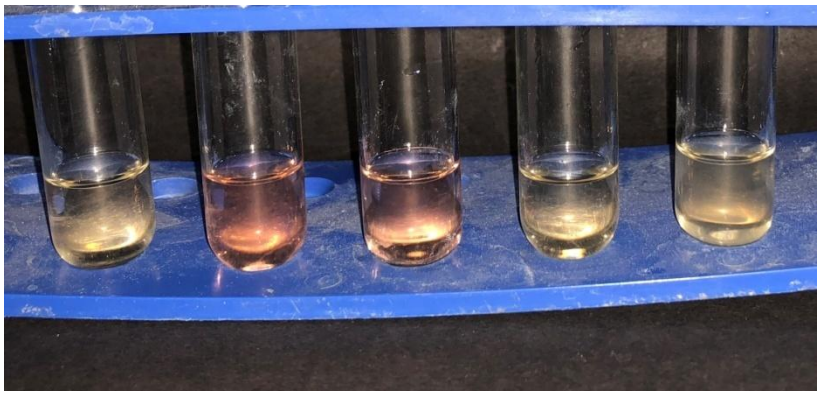


Figure 4.4 IAA production by bacterial isolates.

4.5.2 Phosphate solublization

Phosphate solublization is the solublization of otherwise insoluble form of phosphate i.e. tri calcium phosphate to soluble form visible as creation of halo zones around the bacterial colonies. All seven isolates were screened for phosphate solublization on pikovskaya agar plates. Solublization index was calculated after 3-5 days of incubation. Out of seven isolates, four isolates were able to solublise phosphate and their solublization index was calculated (Table 4.2)

Table 4.2 Solublisation index of bacterial isolates.

Bacterial isolates	Solublization index
HB4	1.10
HB9	**1.61**
HB11	1.08
HB19	**1.28**

Two bacterial isolates HB9 & HB19 showing halo zone on PKV agar plate in Figure 4.5, these isolates showed both IAA production as well as P solublization therefore they were selected for further screening.

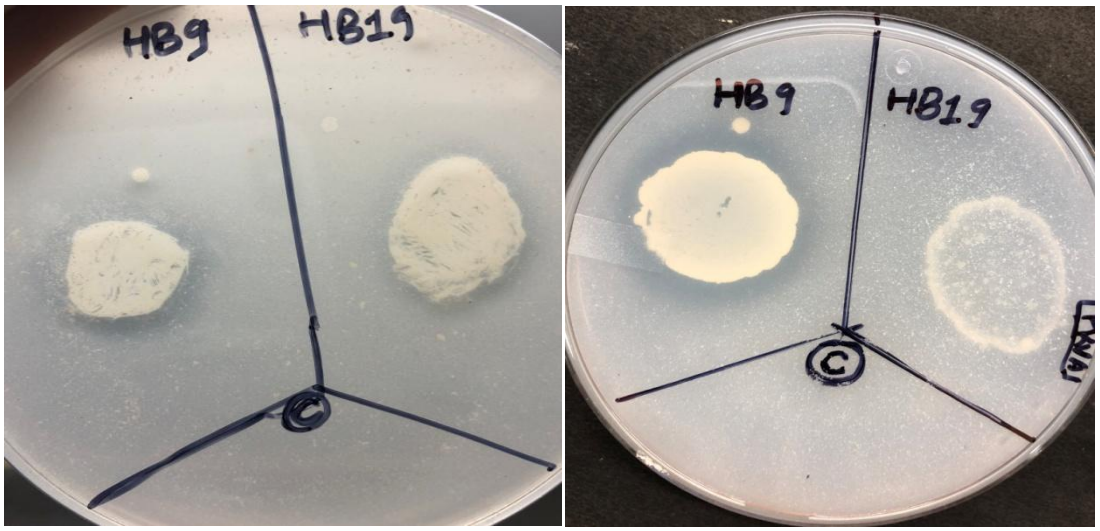


Figure 4.5 Phosphate solublisation by bacterial isolates.

4.5.3 Ammonia production

Ammonia production was determined by using Nessler's reagent. Ammonia produced by the bacteria in peptone broth reacts with the Nessler's reagent and color of the broth changes to yellow orange (Figure 4.6). All the seven isolates were screened for ammonia production, out of which six were able to produce ammonia.

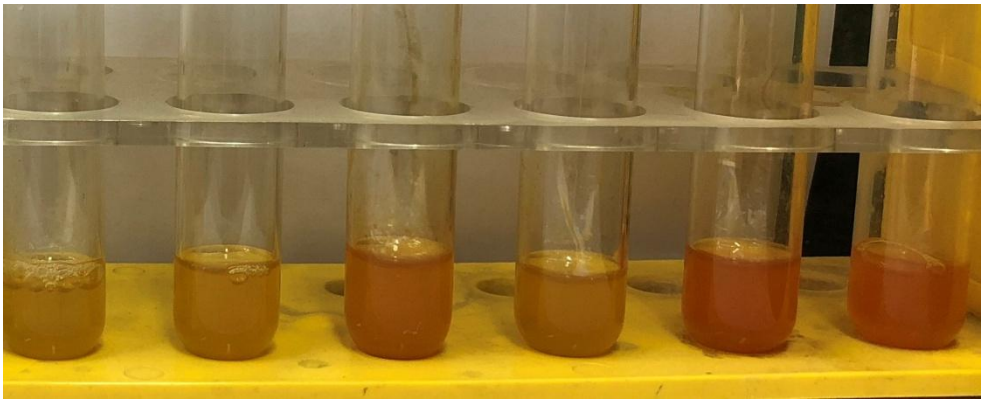


Figure 4.6 Ammonia production by bacterial isolates

4.5.4 HCN production

It is an antimicrobial agent, which promotes plant growth indirectly by decreasing the contact of pathogens with plants, its production can be checked with a color change of filter paper (yellow) dipped in picric acid solution to brown. No color change was observed (Figure 4.7) None of the isolates showed the production of HCN.

Figure 4.7 Screening for production of HCN

4.5.5 Siderophore production

Siderophores are the iron chelating compounds, which can directly or indirectly enhances the plant growth. CAS agar plates were used to check their production. Bacterial colony was inoculated on the plate and after 4 days after incubation, colony showing yellow color around them were selected. Only 1 bacterial isolated showed positive results.

4.6 Quantitative estimation at different salt concentrations

After qualitative screening of all plant growth promoting activities of bacterial isolates, HB9 & HB19 bacterial isolates were finally selected for quantitative estimation of these plant growth promoting characteristics at different NaCl concentrations.

4.6.1 IAA production at different salt concentrations

IAA production was estimated in yeast malt dextrose broth supplemented with 5%, 10%, 15% and 20% salt concentration (Figure 4.8).

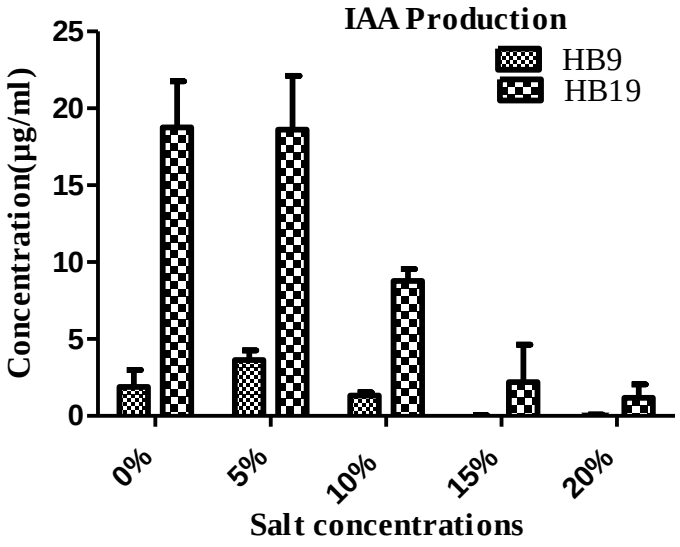


Figure 4.8 IAA production at different salt concentration by HB9 and HB19

From Figure 4.8, it is evident that IAA production is higher by HB19 isolate as compared to HB9 isolate and it gradually reduces with increasing salt concentration. A slight increase in IAA production is observed by HB9 at 5% NaCl concentration as such salt concentration may act as favourable factor in case of halotolerant bacteria.

4.6.2 Phosphate solubilisation at different salt concentrations

Both HB9 and HB19 were subjected to quantitative estimation of Phosphate solublisation in Pikovskaya broth supplemented with 5%, 10%, 15%, 20% NaCl. After incubation with chlorostannous and chloromolybdic acid, the intensity of blue color produced was measured at 600nm. The concentration of solubilised phosphorus in broth at different concentrations was calculated using regression equation of standard concentrations of potassium dihydrogen phosphate.

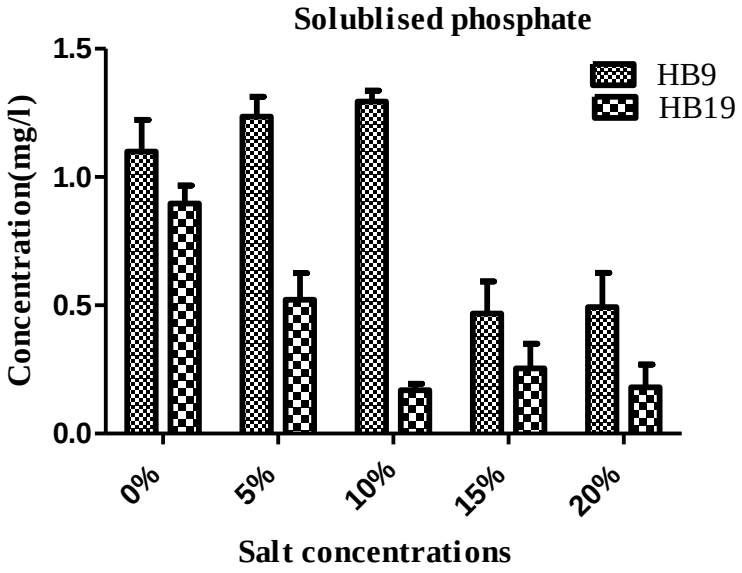


Figure 4.9 Phosphate solublisation at different salt concentrations by HB9 and HB19

Figure4.9 shows that maximum phosphate was solublised at 5% and 10% NaCl concentration by HB9, which is very significant compared to other concentrations. On the other hand, phosphate solublization decreases with increasing salt concentration by HB19.

4.6.3 Ammonia production at different salt concentrations

Peptone broth with different salt concentratons was used for inoculating the bacterial isolates. Nesseler's reagent was used for estimating the ammonia production by bacteria after 24 hours incubation. Color change intensity was measured at 435nm and ammonia concentration was calculated using regression equation of different concentrations of ammonium sulphate

standard solution. Figure4.10 indicates that significant ammonia is produced by both the isolates at 5% NaCl concentration.

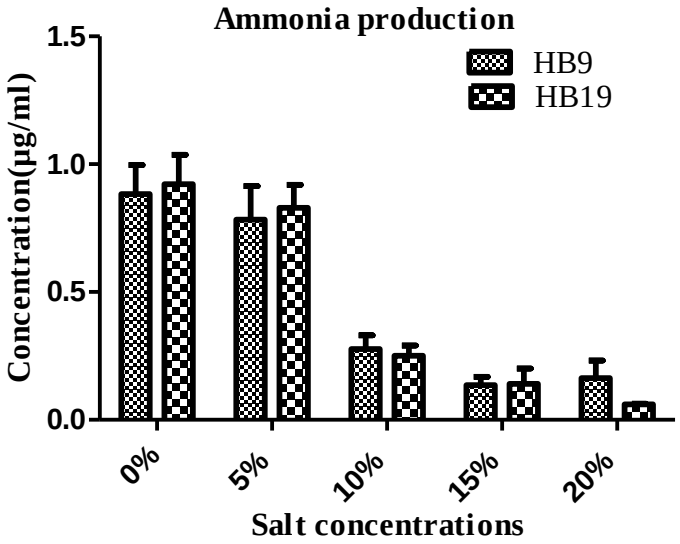


Figure 4.10 Ammonia production at different salt concentrations by HB9 and HB19

4.6.4 Siderophore production at different salt concentration

Siderophore units were quantified for HB9 isolate in broth, after incubation, CAS dye was added and after 20 min. color of the broth becomes lighter in case of bacterial isolate as compared to uninoculated broth. Intensity was taken at 630nm (Figure 4.11)

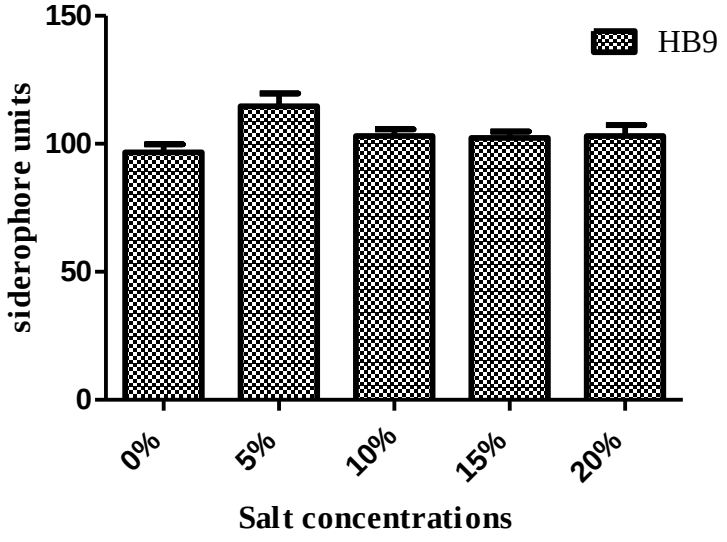


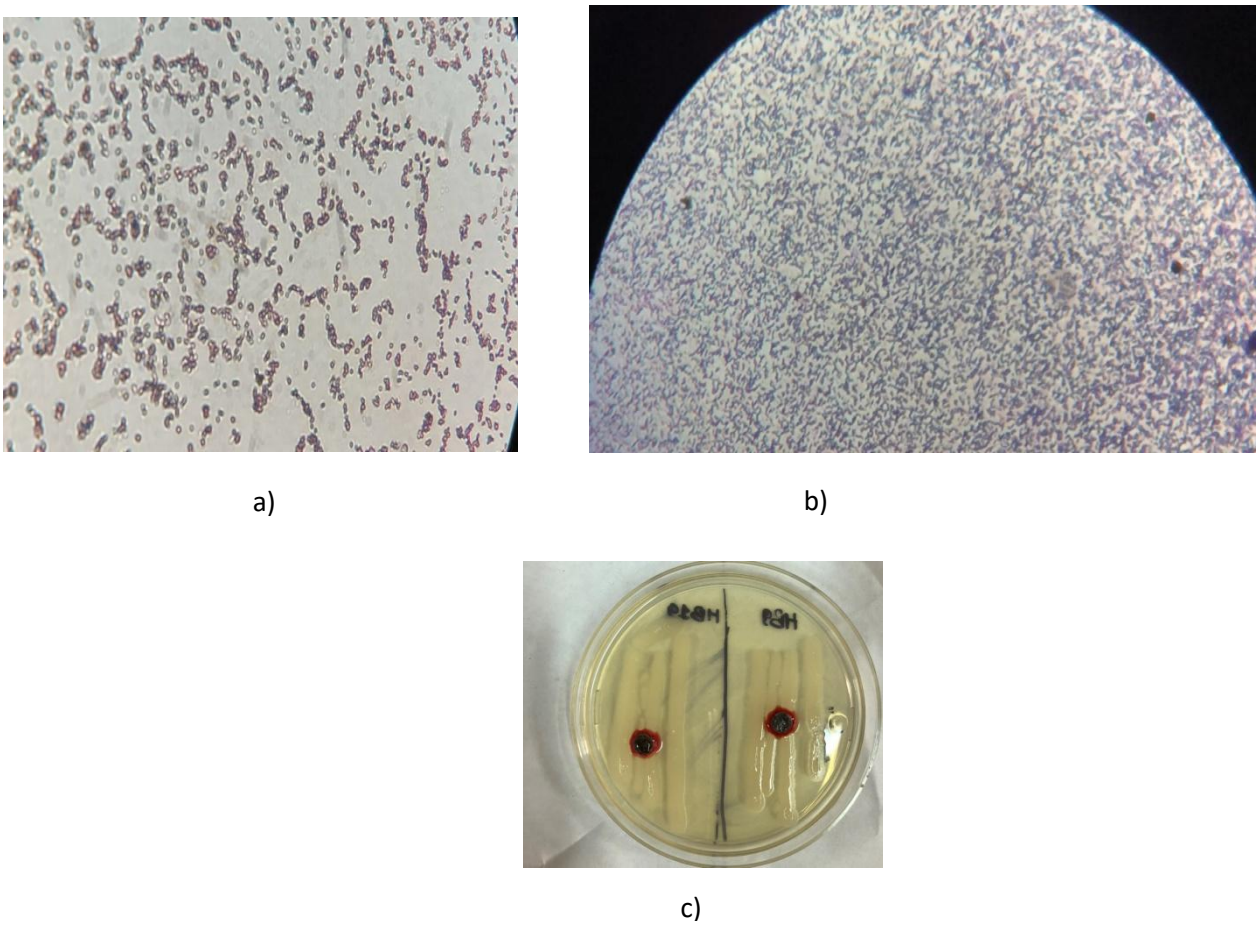
Figure 4.11 Siderophore production by HB9 at different salt concentrations

4.7 Biochemical characterization of isolates

Following biochemical analysis were done on the isolates. Both the isolates are gram positive and showed positive result for nitrate reduction (Figure 4.12)

Table 4.4 Biochemical analysis of HB9 &HB19

	HB9	HB19
Gram staining	**+ve**	**+ve**
Catalase test	**-ve**	**-ve**
Oxidase test	**-ve**	**-ve**
Nitrate reduction test	**+ve**	**+ve**



a) b)

c)

Figure 4.12 Biochemical analysis of HB9 and HB19: a) gram staining of HB19 b) gram staining of HB9 c) nitrate reduction of HB9 and HB19

4.8 Physicochemical analysis of soil sample used in nursery experiment

Soil analysis was done for determining the concentration of major components of soil.

Table 4.5 Soil analysis

Physicochemical characteristic	Values
pH	8.1
Available phosphorus	27.8 mg/kg
Organic carbon	0.264 %
Nitrogen	0.046 %

4.9 Nursery experiments

To study plant growth promotion by the isolates in vivo, nursery trials were set up in plastic bags. Sorghum seeds were inoculated with these isolates HB9 & HB19 with a sugar solution and gum acacia mixture as explained in chapter 3.

3-4 coated seeds were sown in each bag. Total eight treatment were set as follows and growth factors were studies for all the treatments (Figure 4.13)

- CONTROL without salt stress
- CONTROL with salt stress
- HB9 without salt stress
- HB9 with salt stress
- HB19 without salt stress
- HB19 with salt stress
- HB9+HB19 without salt stress
- HB9+HB19 with salt stress

75mm salt stress was maintained in half of the bags by irrigating with 75 mM NaCl in water once a week.

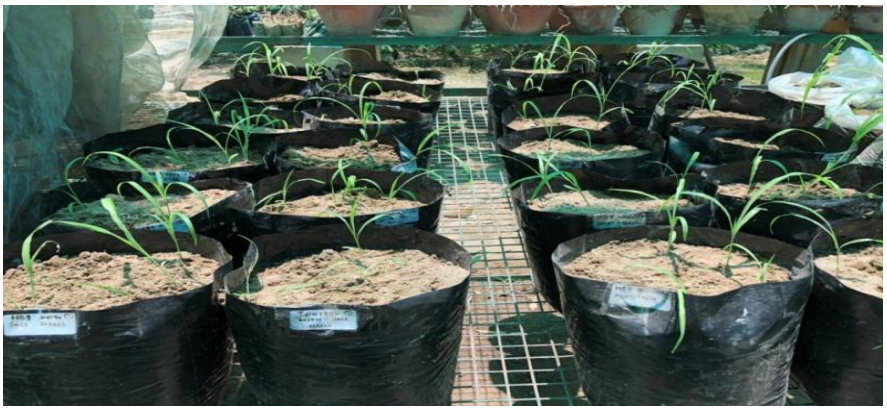


Figure 4.13 Nursery trials on sorghum plants

4.9.1 Electrical conductance and pH of soil after the experiment.

Electrical conductance was in the range of moderately saline for salt stressed soil and pH was slightly alkaline

Table 4.6 pH and electrical conductance of soil samples

Soil sample(in pots) of each treatment	pH	Electrical conductance (dS/m)
CONTROL without salt	8.1	0.36
CONTROL with salt	8.3	2.36
HB9 without salt	7.6	0.30
HB9 with salt	8.2	2.53
HB19 without salt	8.0	0.43
HB19 with salt	8.4	2.92
HB9+HB19 without salt	7.8	0.36
HB9+HB19 with salt	8.6	2.63

4.9.2 Plant growth promotion by isolates

Three plants per treatment were selected for measuring the growth parameters. Control plants with no bacterial inoculums which were given 75mM salt stress were significantly affected as there was decrease in shoot and root length due to salinity stress (Figure 4.14).

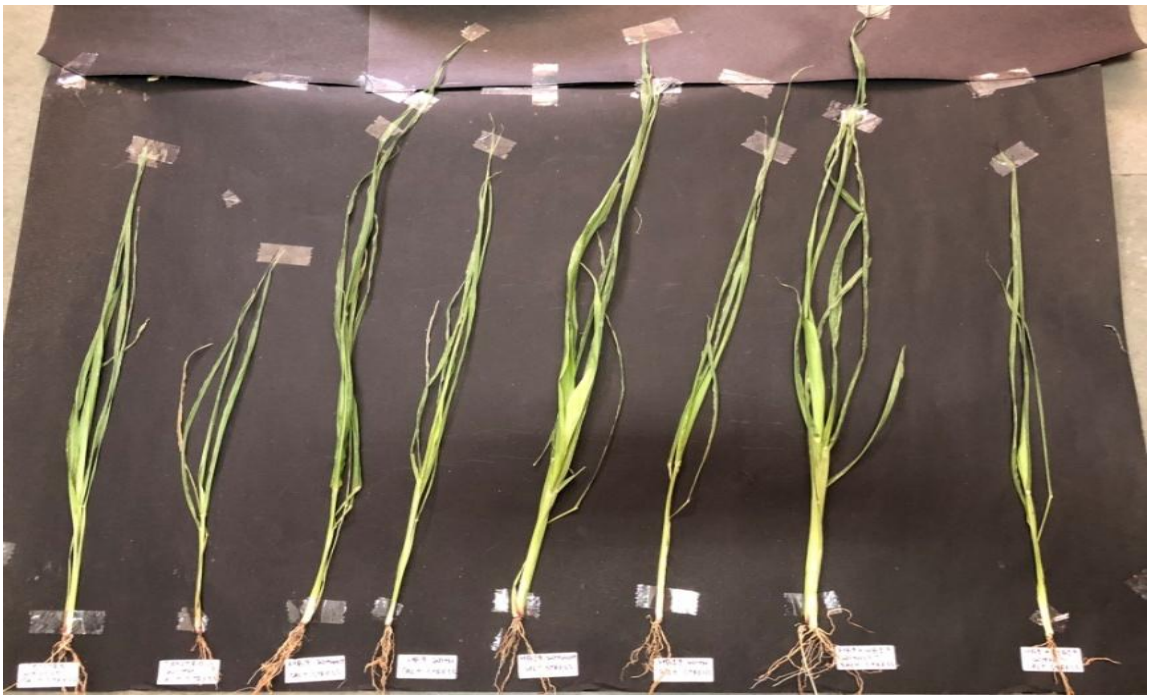


Figure 4.14 Comparison of shoot length and root length of control and treated plants

4.9.3 Growth parameters

Root and shoot length, fresh weight and dry biomass was determined for all differently treated as well as control plants. All data were measured in triplicates.

4.9.3.1 Root length

The inoculated plants have significant rise in root length as compared to the control plants in both salt stressed as well as without salt conditions. The roots of these plants were more healthy. Among the bacterial treatments, root length of the plants treated with the consortium increased more as compared to the individual treatment (Figure 4.15).

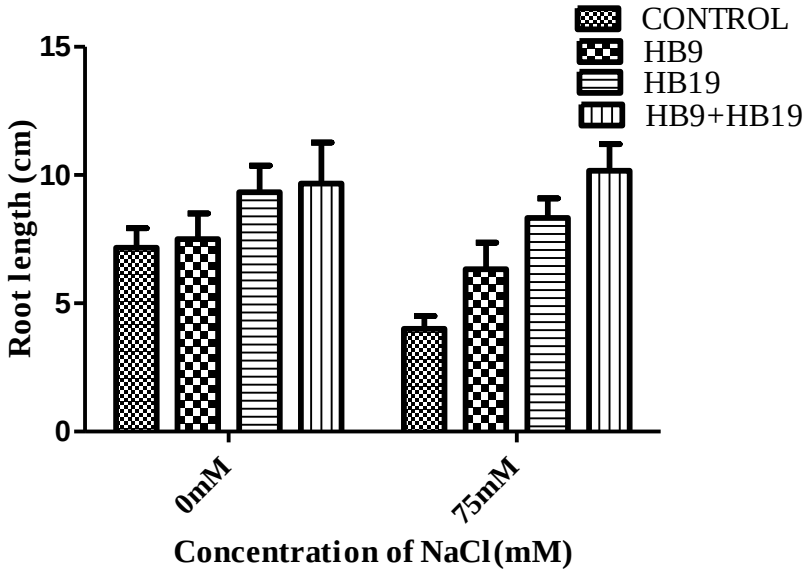


Figure 4.15 Root lengths of plants of different treatments

Table 4.7 Two way ANOVA for root length parameters

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F
Interaction	3	10.2	3.399	3.296*
Bacterial isolates	3	67.45	22.48	21.8****
Concentration	1	8.76	8.76	8.495*
Residual	16	16.5	1.031	

(*) P<0.05, (****) P<0.0001

Two way ANOVA indicates that there is significant variation among the treatments. It is evident that there is highest increase in root length of plants treated with consortium of bacteria. However there is significant increase in root length of plants treated with individual bacteria too as compared to control plants (Table 4.7)

4.9.3.2 Shoot length

Following trend as shown in Figure 4.16 was observed in shoot length. Treated plants have significantly increased shoot length as compared to the control plants in the respective salt concentrations.

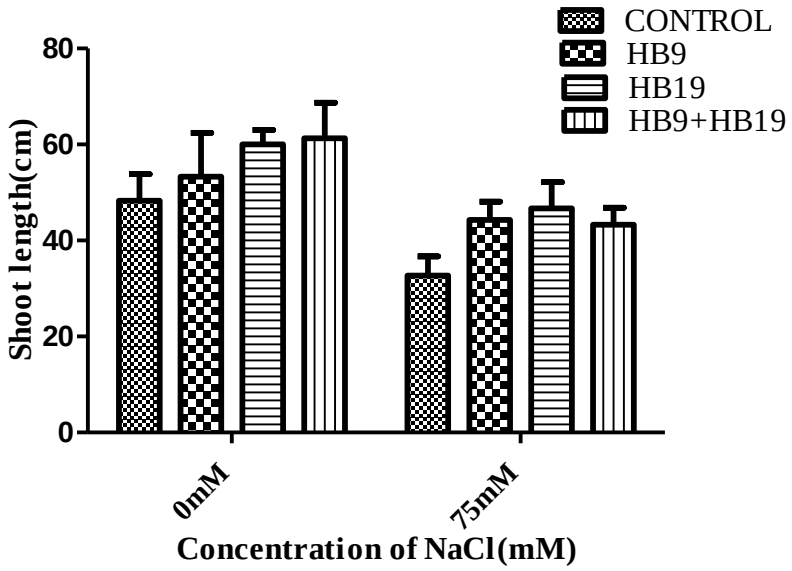


Figure 4.16 Shoot length of plants for different treatments

Table 4.8 Two way ANOVA of shoot length

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F
Interaction	3	66.33	22.11	0.7094
Bacterial isolate	3	611.5	203.8	6.540**
Concentration	1	1176	1176	37.73****
Residual	16	498.7	31.17	

(**) P<0.01, (****) P<0.0001

ANOVA indicates that there was no significant difference among the treatments. But there was significant difference among the factors. The plants treated with consortium of bacteria show maximum shoot length without salt stress. However the plants treated with individual bacteria also increase shoot length as compared to non inoculated control plants in both treatments (Table 4.8)

4.9.3.3 Fresh root weight

Fresh root weight was also increased in treated plants as observed (Figure 4.17). plants treated with HB9 isolate had more root weight as compared to the plants treated individually with HB19 at salt stress as well as at 0mM salt concentration.

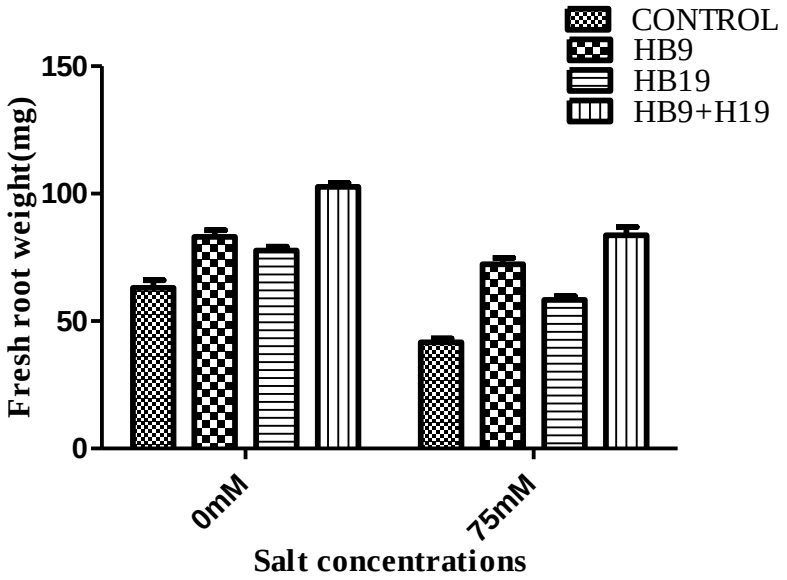


Figure 4.17 Fresh root weight of plants for different treatments

Table 4.9 Two way ANOVA of fresh root weight

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F
Interaction	3	100.5	33.49	6.378**
treatment	3	5282	1761	335.4****
conc.	1	1855	1855	353.3****
Residual	16	84.00	5.250	

(**) P<0.01, (****) P<0.0001

From ANOVA table, it is concluded that there is significant difference among the treatments as well as factors. HB9 inoculated plants showed fresh root weight was significantly increased under salt stress as well as without salt stress. But maximum increase in fresh root weight is seen in plants inoculated with consortium of HB9 & HB19 bacteria (Table 4.9)

4.9.3.4 Fresh shoot weight

Fresh shoot weight was observed to increase in plants treated with the consortium of isolates. at the respective salt concentrations, there was significant incease in shoot biomass of treated plants as comapred to the control plant.

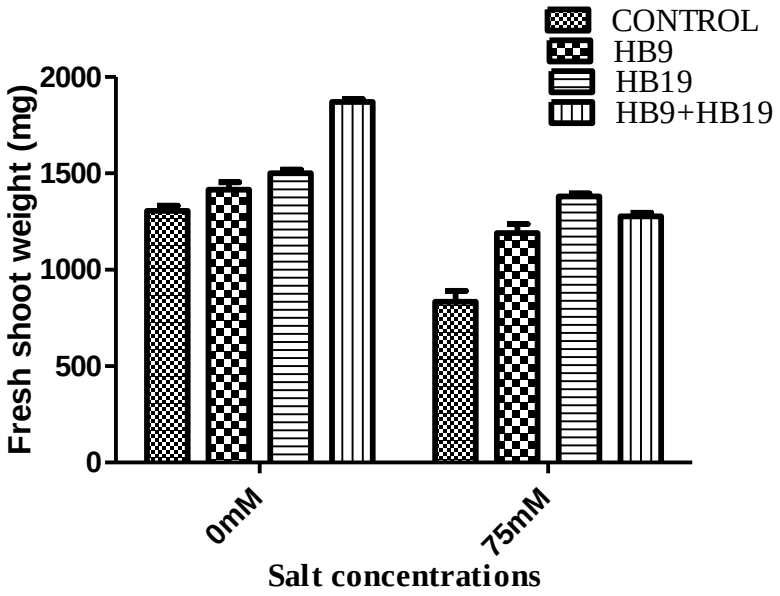


Figure 4.18 Fresh shoot weight of plants for different treatments

Table 4.10 Two way ANOVA for fresh shoot weight

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F
Interaction	3	213874	71291	65.50****
treatment	3	831354	277118	254.6****
conc.	1	745890	745890	685.3****
Residual	16	17415	1088	

(****) P<0.0001

Two way ANOVA indicates that there is significant difference among the treatments. Control as well as bacterial inoculated plants without salt stress have more fresh shoot weight than that of salt stressed plants. However, plants inoculated with individual and consortium of bacteria show increased fresh shoot weight as compared to control plants (Table 4.10).

4.9.3.5 Dry root weight

Plants were incubated at 65°C for 2 days in separate paper bags. Dry weight was measured for each root. Dry root weigth reduced at 75mm salt concentration but among the bacterial treatments , at both salt concentration level, treated plants were observed to have more dry root biomass than the non treated plants.

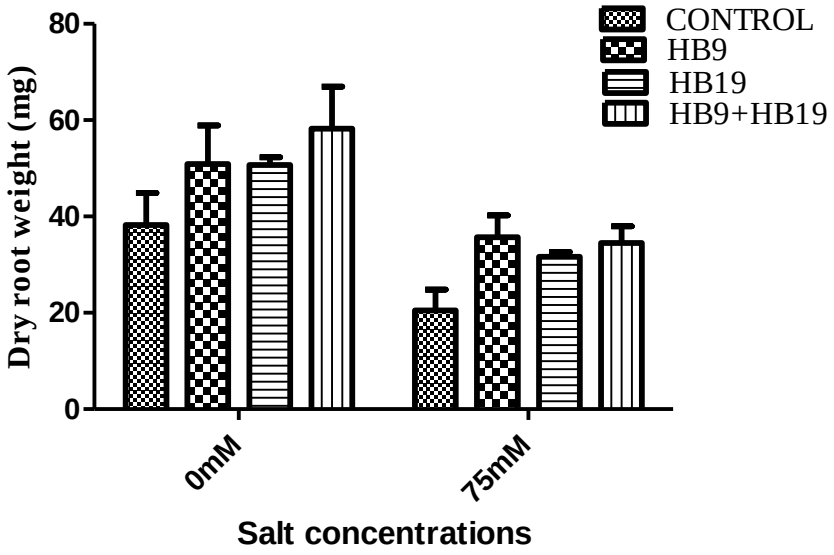


Figure 4.19 Dry root weight of plants for different treatments

Table 4.11 Two way ANOVA of dry root weight

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F
Interaction	3	58.02	19.34	0.6484
treatment	3	993.8	331.3	11.11***
conc.	1	2155	2155	72.24****
Residual	16	477.2	29.83	

(***) P<0001, (****) P<0.0001

ANOVA table indicates that there is no significant difference between interactions. But there is a significant variation among the factors. Increase in dry root weight is seen in plants treated with bacteria as compared to non treated control plants (Table 4.11).

4.9.3.6 Dry Shoot weight

Dry shoot weight was measured after drying the plants. Dry shoot weight was observed to decrease in salt stress. The bacterial treatments significantly increased dry shoot weight of plants as compared to respective control plants.

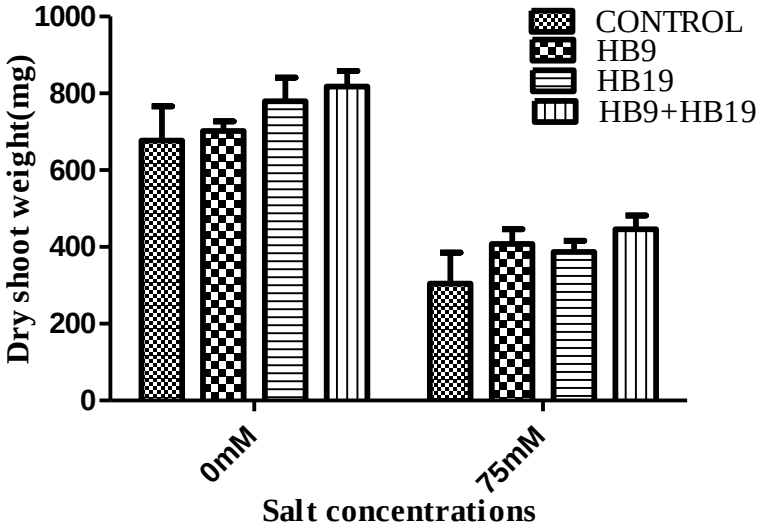


Figure 4.20 Dry shoot weight of plants of different treatments

Table 4.12 Two way ANOVA for dry shoot weight

Source of Variation	Degree of freedom	Sum-of-squares	Mean square	F
Interaction	3	8509	2836	0.9363
treatment	3	62799	20933	6.911**
conc.	1	768096	768096	253.6***
Residual	16	48465	3029	

(**) P<0.01, (***) P<0001

Two way ANOVA indicates that there is no significant difference between interaction. Dry Shoot weight of plants is much affected by salt stress, but there is significant increase in shoot biomass is seen in treated plants as compared to non treated plants (Table 4.12)

4.10 Total P uptake by shoots of plants

Acid digests of shoot sample were used to estimate P uptake by plants, vanadomobdate solution was added and color changes to yellow. Standard regression curve of potassium dihydrogen phosphate was plotted and concentration of total P was measured.

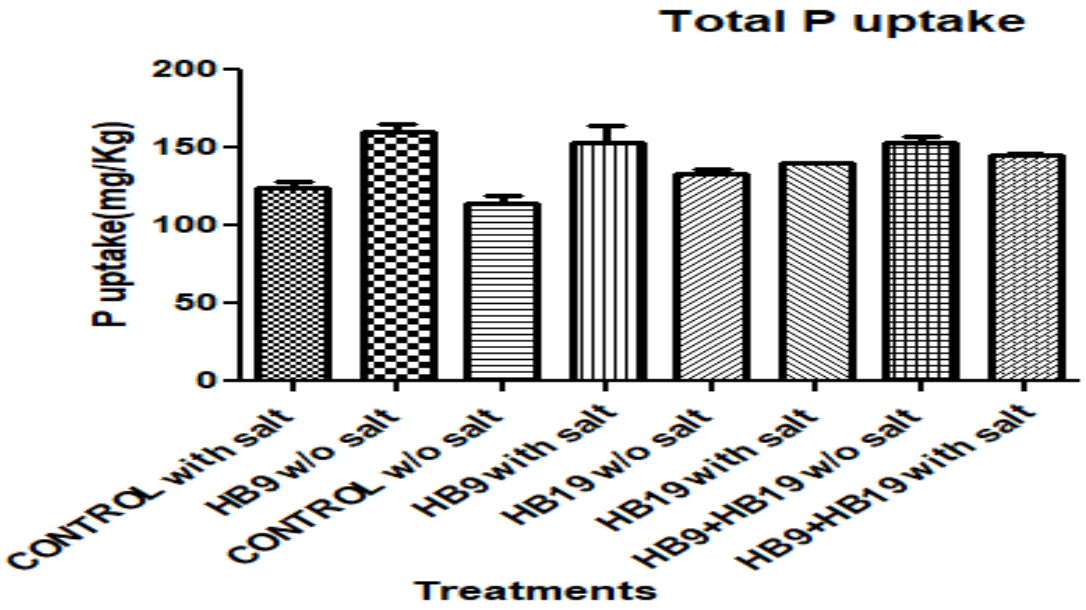


Figure 4.21 Total P uptake by shoots of plants

From Figure 4.21, it can be concluded that plants inoculated with HB9 shows maximum P uptake.

4.11 Molecular characterization of bacterial isolates

4.11.1 Isolation of genomic DNA of bacterial isolates

Agarose gel electrophoresis was done for qualitative estimation of DNA. 1% gel was made for visualizing the DNA bands and the gel was allowed to run for 60 minutes. After which it was visualized in Gel doc, under UV light.

Quantification of DNA was done by using nanodrop. It also gives the purity of the product by giving 260/280nm ratio. The absorbance is taken at 260/280 because nucleic acids i.e. DNA and RNA absorb at this region while proteins absorb at 280nm.

4.11.2 Amplification of 16S rDNA

PCR was done to amplify 16S rDNA region of isolated genomic DNA of both the isolates. Since 16s rDNA is a highly conserved region in prokaryotes, it is used for the identification and classification of bacteria. Therefore, it is also known as a reliable molecular clock.

After the amplification, the concentration of amplified products was also determined by using nanodrop.

Table 4.13 Quantitative estimation of DNA

Sample	Concentration(ng/µl)	Absorbance(260/280)
HB9	**313.4**	**1.71**
HB19	**210.7**	**1.7**
Purified Amplified HB9	**118**	**1.8**
Purified Amplified HB19	**102**	**1.8**

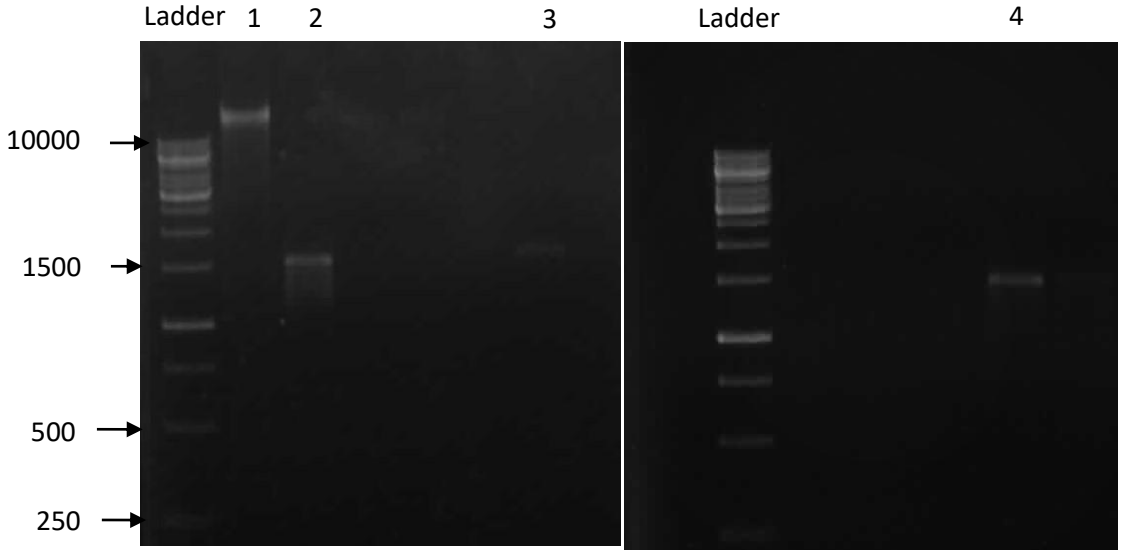


Figure 4.22 Bands showing 1) Genomic DNA of HB9, 2) Amplified product of HB9, 3) Genomic DNA of HB19 4) Amplified product of HB19

4.11.3 16S rRNA sequencing

Purified samples were sent to First Base Asia , Malaysia. The results of sequencing for both the bacterial isolates were :

HB9

CTTGCTAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAA
CCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCG
GCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGG
GTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCAC
CTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
TGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGA
AATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA

ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGC
TGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
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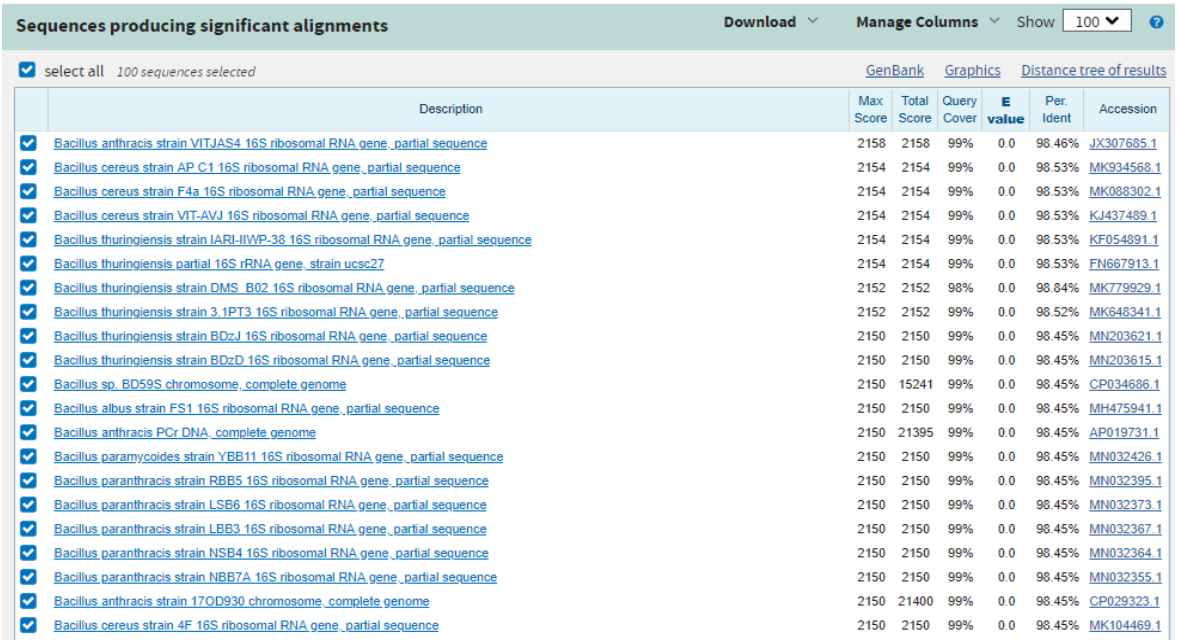

Sequences producing significant alignments Download Manage Columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus anthracis strain VITJAS4 16S ribosomal RNA gene, partial sequence	2158	2158	99%	0.0	98.46%	JX307685.1
Bacillus cereus strain AP C1 16S ribosomal RNA gene, partial sequence	2154	2154	99%	0.0	98.53%	MK934568.1
Bacillus cereus strain F4a 16S ribosomal RNA gene, partial sequence	2154	2154	99%	0.0	98.53%	MK088302.1
Bacillus cereus strain VIT-AVJ 16S ribosomal RNA gene, partial sequence	2154	2154	99%	0.0	98.53%	KJ437489.1
Bacillus thuringiensis strain IARI-IIWP-38 16S ribosomal RNA gene, partial sequence	2154	2154	99%	0.0	98.53%	KF054891.1
Bacillus thuringiensis partial 16S rRNA gene, strain ucsc27	2154	2154	99%	0.0	98.53%	FN667913.1
Bacillus thuringiensis strain DMS_B02 16S ribosomal RNA gene, partial sequence	2152	2152	98%	0.0	98.84%	MK779929.1
Bacillus thuringiensis strain 3.1PT3 16S ribosomal RNA gene, partial sequence	2152	2152	99%	0.0	98.52%	MK648341.1
Bacillus thuringiensis strain BDzJ 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN203621.1
Bacillus thuringiensis strain BDzD 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN203615.1
Bacillus sp. BD59S chromosome, complete genome	2150	15241	99%	0.0	98.45%	CP034686.1
Bacillus albus strain FS1 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MH475941.1
Bacillus anthracis PCr DNA, complete genome	2150	21395	99%	0.0	98.45%	AP019731.1
Bacillus paramycoides strain YBB11 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN032426.1
Bacillus paranthracis strain RBB5 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN032395.1
Bacillus paranthracis strain LSB6 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN032373.1
Bacillus paranthracis strain LBB3 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN032367.1
Bacillus paranthracis strain NSB4 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN032364.1
Bacillus paranthracis strain NBB7A 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MN032355.1
Bacillus anthracis strain 17OD930 chromosome, complete genome	2150	21400	99%	0.0	98.45%	CP029323.1
Bacillus cereus strain 4F 16S ribosomal RNA gene, partial sequence	2150	2150	99%	0.0	98.45%	MK104469.1

Figure 4.23 BLASTN was performed to find related taxa for HB9 sequence

HB19

AGGAGGGGGGGCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTT
ATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACT
GGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGG
TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTA
GTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAA
CCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGA
GGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGT

ACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC
TCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGG
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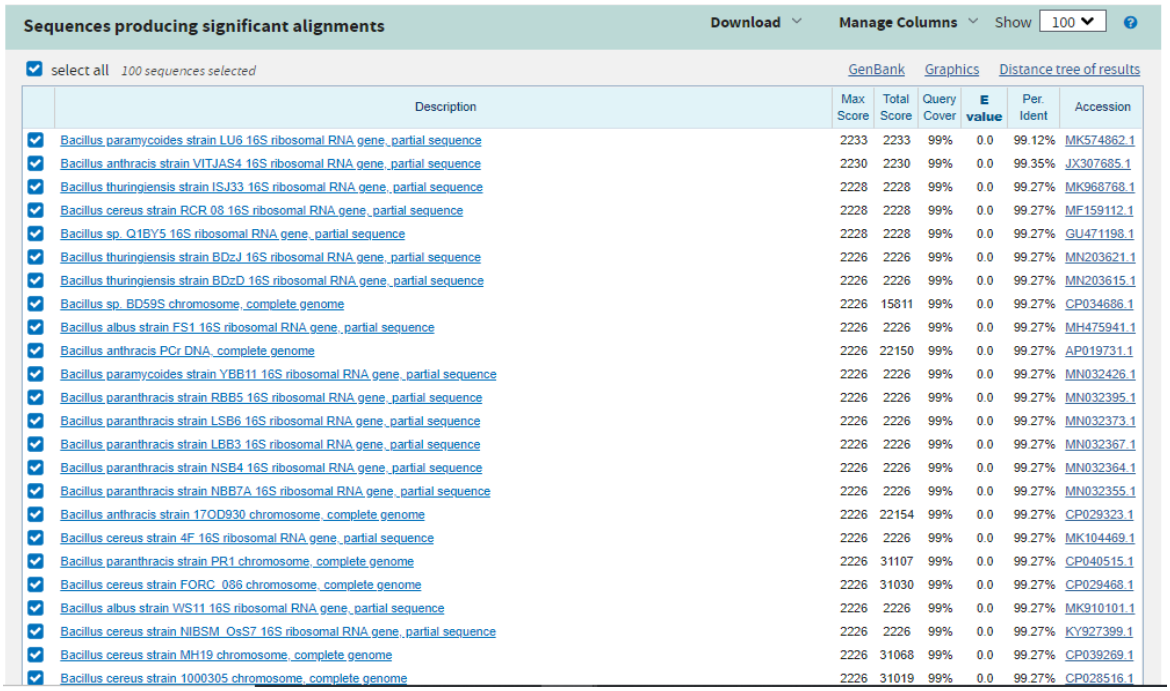

Figure 4.24 BLASTN was performed to relate sequence of HB19

MultAlin was done to align the sequences of these two isolates and other bacterial species related to these taxa to construct a phylogenetic tree showing related taxas of both the isolates.

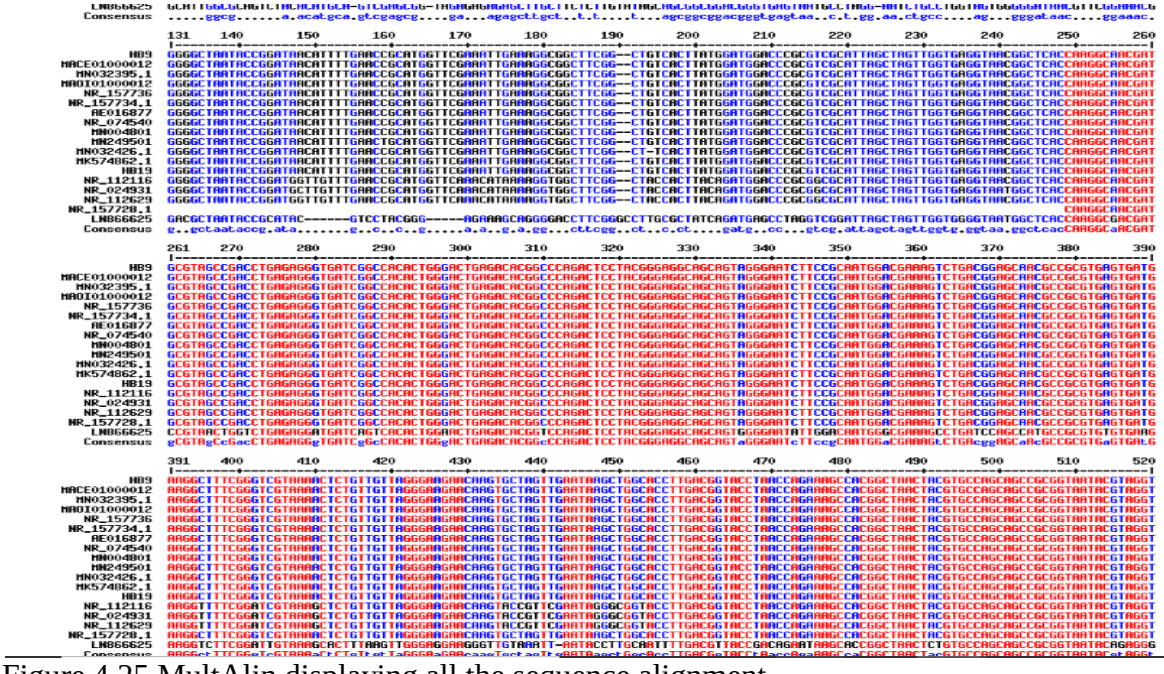
Figure 4.25 MultAlin displaying all the sequence alignment

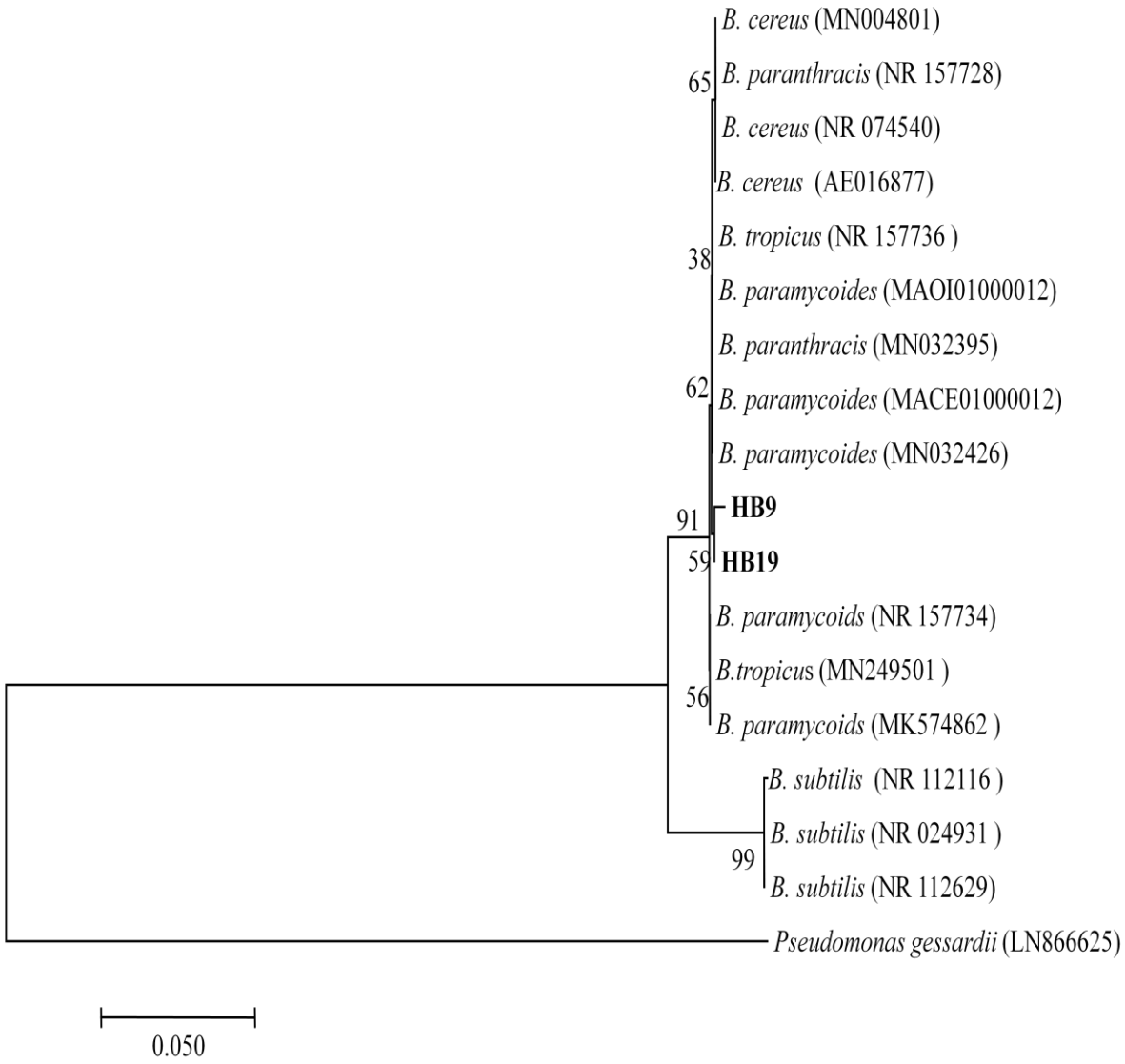

Figure 4.26 Phylogenetic tree showing related taxa of HB9 &HB19

DISCUSSION

Salinity in soil is a devastating stress in the environment. It causes stunted growth in plants and degradation of soil quality. Sambhar lake is a hypersaline environment and has a wide range of halotolerant microorganisms which can be exploited for their characteristics of producing some useful plant growth promoting metabolites. Many plant growth promoting bacteria were isolated from Sambhar salt lake, mainly they were belonging to Actinobacteria, Firmicutes, Proteobacteria. They grow at optimum range of 2-25% salt concentrations and 6-12 pH range (Sahay *et al*., 2012). Archana and Devendra (2014), observed that bacterial isolates of Sambhar lake have salt requirement of 3-15%. Under 200mM salt stress, halophilic bacteria inoculated wheat showed growth rate increase by 62.2% to 78.1%. Furkan orhan (2016), observed that 44% of bacterial strains produced IAA, 28% were nitrogen fixing only one was able to solublise phosphate. *Bacillus, Halobacillus, Halomonas* were the major species having PGP attributes . Co-inoculation of two bacterial species *B. subtilis* and *Arthrobacter spp*. in wheat resulted in alleviation of adverse effects of salt stress (Upadhyay et al., 2011).

Two bacteria were isolated in present study HB9 & HB19 which showed tolerance up to 20% salt. They produced singnificant amount of IAA at different salt concentrations. IAA is an important auxin for plant growth. They were also able to solublise phosphate and produce ammonia and siderophores**.** In nursery trials, significant rise in growth parameters was observed using two way ANOVA for each parameter. Salt stressed control plants at 75mM salt stress were adversely affected, as compared to the plants treated with bacterial isolates at respective salt concentration.

CONCLUSION

Saline soil is widely distributed across the globe. In India, arid and semi arid regions of Gujrat and Rajasthan are are adversely affected by soil salinity. Microbes in soil which are useful for the plant growth by secreting some metabolites also gets affected by high accumulation of salts. Their metabolic activities gets reduced due to oxidative stress caused by high salt concentration. Plant growth and agricultural yield gets reduced due to high salt accumulation in soil.

Sambhar salt lake is a soda lake in Rajasthan, it inhabits many halotolerant microbes which can be beneficial to the environment. Sample collected from this lake had alkaline pH and electrical conductance of 3.6 dSm^{-1}. Total of 22 bacterial isolates were isolated on complex halophilic agar plates. They were screened for salt tolerance up to 20% salt concentration.

Most efficient halotolerant isolates were selected for further determination of plant growth promoting attributes. Both the bacterial isolates were gram positive. They showed negative results for catalase and oxidase. Nitrate reduction test was positive for both isolates.

HB9 and HB19 were the most effective halotolerant PGPB. HB19 produced 20µ/ml IAA in L-tryptophan supplemented broth. Solublization index of HB9 and HB19 was 1.61 and 1.28 repectively. Ammonia and Siderophore production was observed by both the isolates.

Nursery trials were done by inoculating the sorghum seeds with bacterial inoculums. Plants were treated with individual inoculums as well as consortium of the two isolates at 0mM

And 75mM salt concentrations. Salt stress was maintained by irrigating with water having 75mM salt concentration once a week. After harvesting growth parameters were measuresd and two way ANOVA was done on each parameter, a significant rise in root and shoot length, dry biomass, fresh weight in plants treated with these isolates as compared to the control plants at both the salt concentrations.

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study of effect of NaCl concentration on enzyme

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