Evaluation of Endophytic Bacteria for Growth and Stress Tolerance of *Trigonella Foenum-Graecum* (Fenugreek) Under Saline Stress Conditions

Thesis

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By

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C E R T I F I C A T E

This is to certify that the thesis entitled "**Evaluation of endophytic bacteria for growth and stress tolerance of *Trigonella foenum-graecum* (Fenugreek) under saline stress conditions"**submitted in partial fulfillment of the requirements for the degree of **Master of Science** with major in **Microbiology** of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bonafide* research carried out by **Mr. Naveen k** Id. No. **51043** under my supervision and no part of the thesis has been submitted for any other degree or diploma. The assistance and help received during the course of this investigation have been acknowledged.

13/08/2018

Pantnagar
August, 2018

(Lakshmi Tewari)
Chairperson
Advisory Committee

C E R T I F I C A T E

We, the undersigned, members of the Advisory Committee of **Mr. Naveen k** Id. No. **51043,** a candidate for the degree of **Master of Science** with major in **Microbiology** agree that the thesis entitled "**Evaluation of endophytic bacteria for growth and stress tolerance of *Trigonella foenum-graecum* (Fenugreek) under saline stress conditions"**may be submitted in partial fulfillment of the requirements for the degree.

13/08/2018

(Lakshmi Tewari)
Chairperson
Advisory Committee

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Member

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Introduction

One of the major challenges of 21st century is to feed the ever growing population which is estimated to reach 10 billion by 2050(www.unfpa.org). Unfortunately, global agricultural production is under immense stress by both biotic and abiotic stresses causing major damage to food security. Anthropogenic activities play a major role in deterioration of natural resources converting them into uncultivable lands. Accordingly, about 5.2 billion hectare of fertile land is affected by salinity, erosion and soil degradation out of which salt stress alone accounts for 50%; thus creating gamut of problems for farming community around the world (Riadh *et al*., 2010; Ruan *et al*., 2010). Over use of fertilizers and exploitation of irrigation facilities are among the many manmade causes that in the formation of saline and alkaline soils (Zhao *et al*., 2007). Any soil in which are among the electrical conductivity(EC) of the saturation extract(ECe) in the root zone exceeds 4 dSm^{-1}(approximately 40 mM NaCl) at 25^0 c and has exchangeable sodium of 15% is called as saline soil. This is the most critical ECe for most of the crop plants and at this condition they exhibit yield reduction phenomenon. (Munns and Tester 2005; Jamil *et al*., 2011).Salt stress is a major problem of arid and semi arid areas but, nowadays it is spreading to dryland areas due to low rainfall and high evaporation rate.(Butcher *et al*., 2016; Ammundson *et al*., 2015). Agriculture is the largest water consuming sector with about 70% of water consumption and it is increasing at an alarming rate due to increase in population (Hightower and Pierce, 2008). Depleting fresh water reserves are adding additional fuel to the problem for sustenance of food production. (Epstein and Bloom, 1980; Flowers and Colmer, 2008; Marris, 2008).Harmful effects of soil salinization and alkalanization include not only deterioration of soil properties, but its immediate effect is also on the ecosystem and biodiversity making them vulnerable.(Cassel *et a*l., 2015; Aoragus, *et al*., 2014). Recent improvements in soil and irrigation facilities have opened the ways for cultivation under marginalized soils but over exploitation of these facilities have also created salt stress related issues to become intense in agricultural soils.(Zahir *et al*., 2008).

Recent reports suggest that such soils are emerging as major limiting factors in performance of every crop worldwide. (Shannon and Grieve, 1999; Bacilio *et al*.,2004). The studies focusing on improving the performance of crops under saline and other abiotic stress conditions has occupied a central importance in recent days. In order to cope up with salt stress have been developed various mechanisms such as physiological, metabolic and

developmental processes that enhance their salt tolerance capacity; these responses are known as acclimatization, phenotypic plasticity, or environmental variation (Hasegawa *et al*., 2000; Debat and David, 2001; Zhu, 2002; Munns and Tester, 2008; Taiz and Zeiger, 2010).There are many strategies to manage salt stress problem in agriculture, including many agronomic strategies such as growing salt tolerant crops, adoption of drip or micro-jet irrigation practices. Adoption of modern crop production technologies such as site specific cultivation (precision farming), inter cropping, phase cropping are also effective strategies against salinity and alkalinity stress. Although, these practices are known to mitigate salt stress but, its implementation is a difficult task due to lack of good quality irrigation water, moreover its cost of establishment makes them unfeasible technologies for resource poor farmers (Munns *et al*., 2002).

In the present context of increasing burden in terms of sustainability and conservation of biodiversity, microorganisms emerge as promising alternatives to resource poor farmers in increasing the crop productivity under challenging conditions. The use of beneficial microbes as an integral component of agricultural practice is a well known technology since ages (Gill *et al*., 2016). One of the well-known category of microbes, known as plant growth promoting rhizobacteria (PGPR) commonly referred as rhizobacteria, consists of bacteria which inhabit rhizosphere and facilitate plant growth under biotic and abiotic stress conditions (Glick, 2014). In spite of all these beneficial activities of rhizobacteria, their field performance is restricted sometimes due to various factors such as soil properties, external environmental conditions.

These limitations force scientific community to discover alternate candidates who can mitigate various stress conditions and promote plant growth in an eco friendly manner. In this context, role of endophytic microorganisms is highly appreciable because of the fact that they are least affected by environmental disturbances and they exert their beneficial effects on plants. The definition of the term 'endophyte' refers to all organisms that, reside inside the plant tissues at particular period of their lifecycle for variable period of time without causing any symptoms of disease to plants (Stone et al*.,* 2000). The discovery of endophytic micro flora was made quite earlier in 1940's but only during the 21st century the significance of this micro flora recognized fully (Heinrich, 1997). They still remain as an unexplored component of biodiversity. Gradual evolution process made them evolve traits of co evolution and adaptation to the plants internal environment without causing any

form of damage to the host plant's that harbors them. They also play a very important role in plant's overall development by protecting against plant pathogens, insect herbivory and are known to produce many novel antimicrobial secondary metabolites (Arnold and Maynard, 2001). They effectively colonize the healthy plant tissues and exert their effect directly on plant (Bacon and Hinton, 2006). The mechanisms employed by plant growth-promoting bacterial endophytes, are almost similar to those used by rhizospheric plant growth-promoting bacteria (PGPB) These include both direct and indirect functions, such as ammonia production (Marques *et al*., 2010), nitrogen fixation (Compant *et al*., 2005), solubilization of mineral phosphate (Verma *et al.,* 2001), siderophore production (Lodewyckx *et al*., 2002), and production of plant hormones (Costacurta and Vanderleyden, 1995). One of the most important strategies in alleviating abiotic stress is decreasing the plant ethylene levels generated in response to various abiotic stresses. The endophytic bacteria are known to express the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves ACC to a-ketobutyrate and ammonia and thereby decreases ethylene levels in host plants (Sessitsch *et al*., 2005) Along with regulating the plant ethylene levels, they also synthesize many phyto hormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins(Sun *et al*., 2009). Salt affected soils are usually deficient in micro nutrients in particularly of iron. As iron is very critical for plant growth and development, its deficiency causes serious damage to the plants. It is observed that in groundnut crop grown in salt affected soils shows calcium induced iron deficiency (Liu *et al*., 2017)**.**Hence in this regard, Stress tolerant PGPB are also known to produce metal chelating structures known siderophores which chelate the insoluble iron and makes available to plants as iron deficiency is the secondary nutrient deficiency in salt affected soils (Kantachote *et al*., 2016).These phytohormones collectively enhance the plant growth by increasing plant height and biomass(Penrose *et al*., 2001).Some of the endophytic strains of *Bacillus, Burkholderia, Enterobacter, Pseudomonas, and Serratia* were found to be very effective in preventing the growth of pathogenic microorganisms under in vivo and in vitro conditions (Hashem *et al*., 2016).While, the endophytic strains which very well versed in conferring the tolerance against abiotic stresses such as drought, heat and salt stress in many agricultural crop plants, they include the strains of the genera *Bacillus, Enterobacter, Pseudomonas, Azotobacter, Arthrobacter, Streptomyces, and Isoptericola.* (Berg *et al*., 2013) .Interestingly, they not only mitigate the stress on the plants in the mean while they also increase plant biomass and height (Sharma *et al*., 2012).The exact

mechanisms by which these bacteria confer abiotic stress is still a hard nut to crack. (Beneduzi *et al*., 2012).Largely remained an unexplored wealth now being considered as a promising tool in sustainable agriculture (Malfanova *et al*., 2011) They are found in almost all plant species including, aromatic and medicinal plants, halophytes etc. (Guimarães *et al*., 2012;Egamberdieva *et al*., 2017).

The mystery behind the unique attributes of the plants potential to synthesize many useful products can be attributed partially to the endophytic organisms present in the. There are many reports which prove that in a microbe-plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antimicrobial and antifungal activities.

Further research in endophyte - plant interactions can give us in depth understanding how they promote plant health and can play a significant role in low input sustainable agriculture applications (Ryan *et al*., 2008).Medicinal and aromatic plants are the plethora of endophytic microorganisms which aid in synthesis of secondary metabolites which in turn provide essential nutrients for their growth. Hence the endophytic bacteria from medicinal plants are known to be highly diverse and perform various functions which aid in plant growth in stress conditions. One such underutilized medicinal plant, *Phyllanthus* sp is a well known to ancient Indian medicine but its cultivation is still in infant stage. Apart from being a pharmaceutical crop, it also harbors diverse range of microorganisms which can play an important role in plant growth promotion under abiotic stress conditions. It consists of nearly 1000 species, spread over the American, African, Australian, and Asian continents (Nathiya *et al*., 2014). Different growth stages of plant are visible among the *Phyllanthus* sp and they have exhibited wide range of metabolic diversity. Most of the species belonging to the genus *Phyllanthus* have been shown to contain different combinations of secondary metabolites which render them with medicinal properties. The major types of bioactive compounds like alkaloids, flavonoids, lignans, phenols, tannins, and terpenes has been isolated from these herbs (Laila and Murtaza, 2015).

Fenugreek (*Trigonella foenum-graecum* L.) a leguminous annual herb belonging to the family *Fabaceae* is cultivated widely in India, Pakistan, Egypt and Middle Eastern countries (Alarcon-Aguilara *et al.*, 1998).Some of the important fenugreek leafy vegeatable growing states are: Rajasthan, Gujrat, Madhya Pradesh, Maharashtra, Haryana,

Punjab, Bihar and Andhra Pradesh (Mehta *et al.*, 2010).India share commanding role in production, consumption, and export of fenugreek in the world. Commonly known as methi it occupies a very important role among all the spices (Kaushik, 2011).

All the parts of fenugreek possess many beneficial attributes which make it an indispensible ingredient in Indian culinary preparations (Ody, 1993). India is known to world for its spices and condiments, being the largest exporter, producer of many spices, among them fenugreek has a special status of calming the largest share in world's export(Petropoulous, 2002).It has many uses such as spice(seed), flavoring agent, medicinal plant, vegetable, fodder and green manure (Acharya *et al.*, 2008). Recently fenugreek cultivation is confronted with low productivity issues to various abiotic stress, among them salinity stress is a major limiting factor in fenugreek production (Tuncturk, 2011).

Objectives: In the view of above, present investigation was carried out with following objectives:

1. Isolation, screening and selection of endophytic bacteria from different regions of Phyllanthus sp for pH and salt stress tolerance.
2. Qualitative and quantitative screening of the selected bacterial isolates for various plant growth promoting traits.
3. *In situ* evaluation of selected endophytic bacterial cultures on the growth and salt tolerance of the leafy vegetable fenugreek under pot culture experiments.

Review of Literature

Soil salinity: A threat to global food security

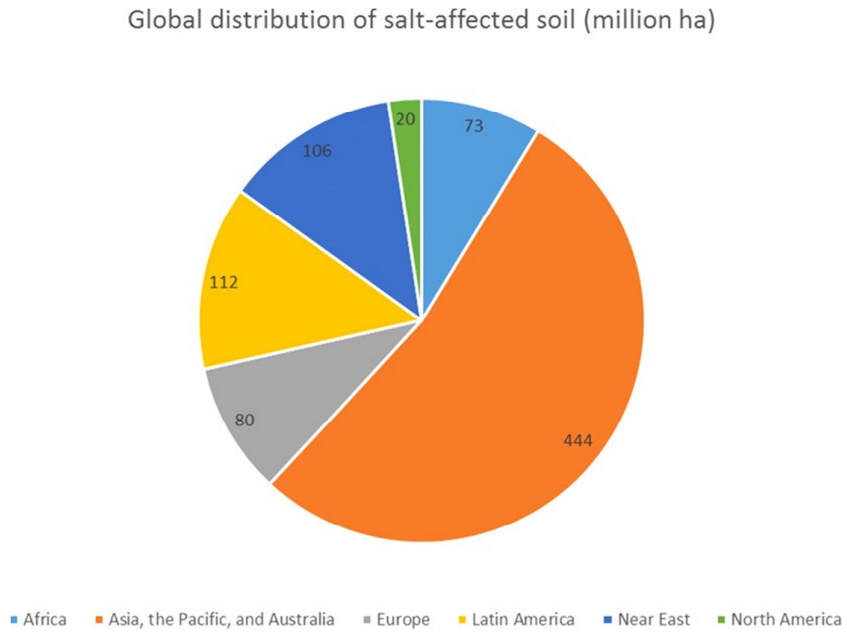
During the recent times there is unprecended increase in the activities which cause the threat to the ecosystem imbalance; on e such example is conversion of agricultural lands into degraded lands which are unfit for cultivation. The Overuse of irrigation facilities have made soils turn into saline or salt affected soils, they are becoming the greatest barriers in crop production. Ever increasing human population, depleting natural resources are emerging as big challenges to agriculture worldwide. These problems collectively have become a threat to food, nutritional and the developmental goals. World's premier organization has released a report jointly with international technical panel (ITPS) on soils "status of the world's soil resources" that has put forth its concerns about the status of soils around the world which are the serious threat of degradation. It also stated that loss of fertile soils due to salinity related issues make the problems of billions of lives vulnerable and plunge them into extreme poverty. About 33 percent land is in the state of degradation worldwide. Rapid urbanization coupled with mismanagement of natural resources has intensified the problems of food production sector. Most of the earlier civilizations ruined because of the fact that misuse of natural resources mainly soil paved the way for their collapse, the ill effects caused by soil salinity and alkalinity are irreversible and needs immediate attention from the scientific community. Recently Qadir *et al.* (2014) have reported that approximately, 2000 hectares of irrigated soil is lost every day due salinity related issues. Salt- affected soils cause damage to 20% of all irrigated lands, causing US$27 billion loss per year. Salt affected soils contribute to 6% of the world's area. Salinity and sodicity problems affect 6.73 Mha of total cultivable land in India.

Distribution of saline soil worldwide and India:

Salt affected soils are spreading at a faster rate to almost all parts of the world, they are now evident in arid, semi arid climate causing lot of damage to agricultural production. Along with geological formations, anthropogenic causes such as, misuse of irrigation facilities, overuse of chemical inputs and other activities responsible for increased salt accumulation in the surface horizon of the soil. Globally most of the salt affected soils are

distributed in Asia, the Pacific, and Australia (444 Mha) and this area is increasing rapidly annually.(Fig:2.1).Other areas are also not exempted from this problem. Latin America also facing a threat from salt affected soils after Asia in terms of area under salt affected soils. In particular, salt stress affected soils are increasing annually in India, in which the highest area is in Uttarpradesh (1368960), followed by Maharashtra (606759), Rajasthan (374942). (Table 2.1).Almost all the states were affected by salt stress, causing a serious implications for crop production.

Figure.2.1. Global distribution of salt-affected soils in million hectares (Mha):



Source: (www.researchgate.net/scientific-contributions)

Table.2.2.Properties of saline, saline–alkali and nonsaline–alkali soils:

Properties	**Saline soils**	**Saline-alkali soils**	**Nonsaline-alkali soils**
Electrical conductivity(dsm^{-1})	**>4.0**	**>4.0**	**<4.0**
pH	**<8.5**	**>8.5**	**>8.5**
Exchangeable sodium percent	**<15**	**>15**	**>15**

Table 2.1.Extent of salt-affected soils India (ha):

State	Saline soils(ha)	Alkaline soils(ha)	Coastal saline soils(ha)	Total(ha)
Andrapradesh	0	196609	77598	274207
Andaman and Nicobar islands	0	0	77000	77000
Bihar	47301	105852	0	153153
Gujarat	1218255	541430	462315	2222000
Haryana	49157	183399	0	232556
Jammu and Kashmir	0	17500	0	17500
Karnataka	1307	148136	586	150029
Kerala	0	0	20000	20000
Maharashtra	177093	422670	6996	606759
Madhya pradesh	0	139720	0	139720
Orissa	0	0	147138	147138
Punjab	0	151717	0	374942
Rajasthan	195571	179371	0	374942
Tamilnadu	0	354784	13231	368960
Uttarpradesh	21989	1346971	0	1368960
West Bengal	0	0	441272	441272
Total	1710673	3788159	1246136	6744968

Source: Central soil salinity research institute, Karnal (Annual report, 2017-18).

Effect of salinity on crop plants:

Agricultural crops exhibit wide range of responses under salt stress conditions. Salinity not only decreases the agricultural productivity of the crop plants but it also affects the soil physico chemical properties and ecological balance of that area (Hu and Schmidhalter, 2002).The characteristic responses of plants to the salinity stress include reduction in the seed germination, plant growth, water and nutrient uptake(Singh and Chatrath,2001; Akbarimoghaddam *et al*., 2011). It also imposes osmotic stress, nutrient

(N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants. One of the major macronutrient deficiency i.e. of phosphorus occurs in salt stress affected soils (Bano and Fatima, 2009).Toxicity of some of the ions such as sodium, chlorine and boron increases in plants under these conditions. Excessive accumulation of sodium ions in the plant cell wall leads to osmotic stress and eventually leads to cell death (Munns, 2002).Higher concentration of salts in soil leads to disturbance in nutrient uptake in plants (Blaylock *et al*., 1994). Other ill effects include reduction in leaf area, chlorophyll content and stomatal conductance, and to a lesser extent through a decrease in photo system II efficiency (Netondo *et al*., 2010) stamen filament elongation, enhancing programmed cell death in some tissue types, ovule abortion and senescence of fertilized embryos. All these developments lead to overall disintegration of plant's growth at physiological, biochemical and molecular levels (Munns and James, 2003; Tester and Davenport, 2003).Decreased performance of plants during salinity conditions is due to loss of turgidity, cell dehydration and Impairment of supply of the photosynthetic assimilates or hormones to the growing tissues which ultimately leads to death of cells (Ashraf, 2004).The adverse effects of salinity on plant development are profound during reproductive phase. It is reported that when wheat plants were exposed to salt levels of 100-175 mM NaCl concentration showed a significant reduction in number of spikelet's, delayed spike emergence. (Munns, 2002)

Existing approaches for salinity amelioration in agricultural soils

Expansion in the area under irrigation and excessive exploitation of water resources paved the way for the conversion of fertile land to salt accumulated land making them unfavorable for cultivation. Many approaches are been employed in mitigation of salinity stress in agricultural soils are categorized as: a) physical approaches b) chemical approaches c) agronomical approaches d) biological approaches. The biological approaches include traditional plant breeding, biotechnological and microbial mediated approaches (Fig 1).

Physical approaches:

Physical methods of amelioration of salt affected soils are very primitive, practiced since ages and include leaching of salts from the top soil to lower depths down below the root zone. This method is applicable in areas where sufficient water is available for leaching the salts to below the root zone (Beltra, 1998). Another method of reclaiming the

salt affected soils is the surface flushing of salts and continuous ponding technique which includes continuous ponding of water on the soil surface as 70% of the soluble salts are present on the soil surface. (Hoffman 1986).However physical methods known to reclaim the salt affected soils are very costly and involve lot of human labor.

Chemical approaches:

This approach mainly relies on the use of chemical amendments in reclaimation of salt affected soils and they are being used frequently in agricultural soils. The common feature of these amendments is that they produce Ca^{+2} ions in sodic soils, directly or indirectly through chemical reactions. Some of the commonly used chemical amendments are either the soluble salts of calcium chloride, mined gypsum, phospho gypsum, iron pyrite, or lime sulphur (Wallace *et al*., 1986). Several other commercial amendments used are the byproducts of certain industries e.g: Pressmud from the sugar industry and organic matter which includes poultry waste, agricultural wastes but the rate of reclamation is very slow with these amendments.(Chand *et al*., 1977).However addition of chemical fertilizers such as calcium nitrate and single super phosphate supply some Ca^{+2} to the soils but they are highly costly and non eco friendly methods of reclaiming the salt affected soils.

Agronomical approaches:

Agronomic approaches offer sustainable solutions for management of salt affected soils such as choice of proper crops, planting dates, planting geometry and irrigation scheduling offer sustainable solutions for management of salt affected agricultural soils. Crop management activities, such as drip irrigation, micro irrigation are also encouraged to minimize the ill-effects caused by irrigation. In order to minimize the spread of dry land salinity, deep rooted trees are encouraged which will prevent the rising water table and movement of salt to soil surface (Manchanda and Garg, 2008). Integrated farming practices such as mixed farming, alley farming, intercropping, precision farming are some of the agronomic approaches to manage the salinity in agricultural soils (Munns *et al*., 2002).Although these approaches can mitigate the salinity stress in soil, but lack of availability of good quality water still prevails. (Munns and James, 2003)

2.5.4 Biological approaches:

Primarily biological approaches focus on employing the salt tolerant crops(Halophytes) in mitigation of salt stress in agricultural soils.(Ma L, Zhang H, Sun L, *et al*.,2012) However, most of the crop plants are glycophytes hence the development of salt tolerant crops by traditional breeding methods became an effective strategy to combat salt stress. (Yamaguchi, T., Blumwald, E., 2005) In this regard many salt tolerant crops were developed and they are successfully cultivated in salt stress affected soils. Benefits of development of salt tolerant crop are many such as they can reclaim the salt affected soils, poor quality irrigation water can be used during their growth period.(Foolad and Chen, 1999). Although, salt tolerant crop plants is well familiarized, but complete understanding at the molecular level is yet to be achieved.(Grover *et al.*, 2003). Development of transgenic salt tolerant crop varieties is well familiarized, also not yet fruitful because of the complex nature of genetic traits controlling tolerance to salt stress (Schubert *et al*., 2009; Dodd and Perez- Alfocea, 2012). The success of transgenic crops is often limited because usually these experiments are performed under controlled conditions and their field success is not impressive. (Yao *et al.,* 2011).Hence field evaluation as a function of yield is a very crucial step in assessing the tolerance levels of plants to salinity conditions (Richards, 1983). Evaluating tolerance to salinity stress is a very difficult task because of variation in sensitivity to salt during the life cycle. For example, in case of paddy, grain yield is much more affected by salinity than the vegetative growth (Khatun and Flowers, 1995). So, the microbial mediated approaches (Plant growth promoting rhizobacteria and endophytic bacteria) are gaining the central attention in almost all remediation programs, due to their versatile nature, diversity and efficiency. (Kohler *et al.,* 2006).(Fig 2.3) Now they have lion share in sustainable agricultural practices.. In this regard microbial mediated strategies play an important role in rectifying the defects of other biological solutions. Thus various biological approaches such as traditional breeding, rhizobacterial and fungal mediated and fungal mediated development of salt resistant transgenic crops and are of endophytic microflora are used for abiotic salt stress tolerance (Fig 2.2)

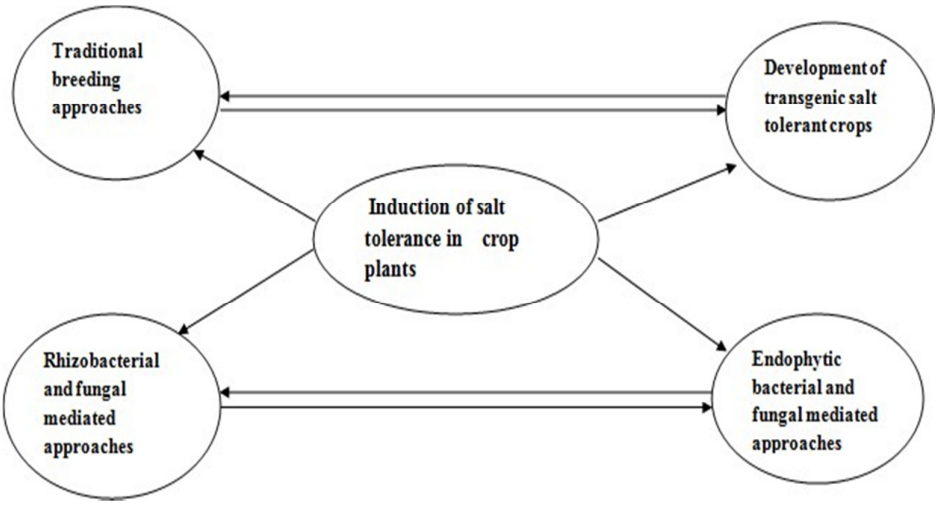


Figure 2.2: Schematic representation of different biological approaches that are used in mitigation of salinity stress in agriculture

2.6 Endophytic microorganisms

Approximately 300,000 plant species are living on the earth and each species is known to habitat one or more kinds of entophytes (Petrini 1991; Strobel and Daisy 2003; Huang *et al*. 2007).Extensive research has been are carried out in order to isolate the endophytic organisms from different parts of various plants, such as roots, stems, leaves, inflorescence of weeds, fruits and important vegetables(Bhore *et al*. 2010; Bulgari *et al*. 2012 Munif *et al*. 2012).Many endophytic bacteria have been isolated from both monocot and dicot such as maize, rice, wheat, beans, groundnut(Miyamoto *et al*. 2004, Peng *et al*. 2006**,** Gangwar and Kaur 2009; Kelemu *et al*. 2011; Lin *et al*. 2012, Rogers *et al*. 2012).Many of the forest trees are also known to inhabit the large number of endophytic microorganisms (Scortichini and Loreti 2007 Krid *et al.* 2010;).

The existence of endophytes has also been confirmed in beets, corn, bananas, tomatoes, and rice roots (Pereira *et al.* 1999, Brown *et al*. 1999; Cao *et al*. 2005,Altalhi 2009).Endophytic microorganisms share a wide range of geographical distribution such as tropical, sub tropical, temperate and coastal mangroves (Janarthine *et al*. 2011). Fungal

endophytes are sometimes observed as antagonistic endophytes commonly associated with medicinal plants and weeds. (Ting *et al.* 2009).It took some time to the scientific community to define endophytic microorganisms, Now they are defined as group of microorganisms which colonize living, internal tissues of plants without causing any immediate negative effects on plants.

Bary was the first person to give the term endophyte in 1886 for the group of microorganisms Bary for microorganisms such as, fungi, yeast, and bacteria which colonize the internal plant tissues. De Bary(1884). Soil microorganisms can become endophytic microorganisms after successful penetration and colonization inside the plant tissues.(Galippe 1887). Earlier belief regarding the endophytic microorganisms that they are as a result of contamination during the isolation process completely disproved in further studies.(Smith 1911).Latest definition of endophytic microorganisms is that all organisms inhabiting the different internal parts of plants, including seeds. (Posada and Vega ,2005) .

Earlier studies on endophytes focused on abundance, species richness but not on their interactions (Tan and Zou 2001). Intensive research on endophytes took place between 1933 and 1989,which was focused on different grass species (Sampson 1938, Latch *et al*. 1985, Saha *et al*. 1987 ,White 1987,Clay and Schardl 2002).Along the timeline, research focused on endophytes of woody plants such as coniferous tress and deciduous trees (Petrini and Petrini 1985; Petrini 1991).After examining the distribution of endophytes in the plant internal tissues, next major task was to isolate them from internal tissues without any difficulty, in this regard many workers introduced several isolation protocols. Schulz and coworkers in 1998 introduced leaf imprint as a new method for checking the isolation protocols, aiming to eliminate epiphytic organisms (Schulz *et al.* 1998). Sánchez and Márquez (2008) used this approach as an excellent sterilization method for isolation of endophytes from one kind of grass (*Dactylis glomerata* L.). The method has been further developed (Suryanarayanan and Kumaresan 2000, Arnold *et al.* 2001).Currently, a substantial body of research on endophytes focused on the methods of isolation, biodiversity, secondary metabolites, and especially mechanisms of the interaction between the endophyte and the host.

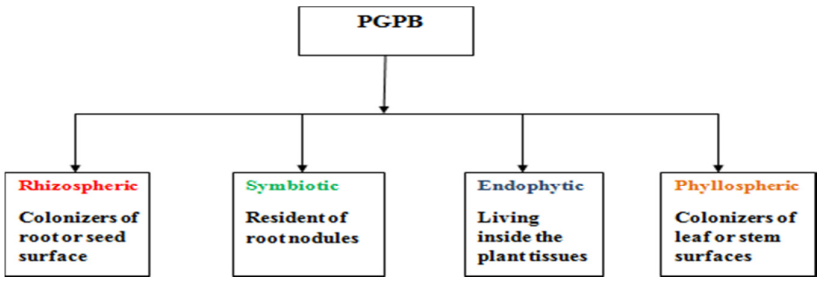


Figure 2.3. Classification of plant growth promoting bacteria (PGPB)

2.7 Bacterial endophytes and abiotic stress mitigation in plants:

Bacterial endophytes are better placed when compared with their counterpart rhizospheric bacteria because of their direct contact with the internal tissues of the plants and therefore they exert direct beneficial effects to plant. However, rhizospheric bacteria can also enter plant tissues and colonize successfully (Hallmann *et al*., 1997). They are known as subset of rhizospheric bacteria (Germida *et al*., 1998; Marquez-Santacruz *et al*., 2010).The rhizospheric region is characterized by high microbial diversity and also a very competitive environment for diverse microbial communities to establish (Raaijmakers *et al*., 2002). So, these conditions exert pressure on microorganisms to be either beneficial or pathogenic to plants although some remain neutral (Haas and Keel, 2003). Some of the key traits that are involved in microbial colonization of plant roots include production of polysaccharides and certain other secondary metabolites (Holguin, 1993; Bashan and 1995; You *et al*., 1995). Bacterial endophytes are considered one step advanced from rhizospheric bacteria and improve the plant performance under hostile conditions (Ali *et al*., 2014). Recently there is an increase in interest in endophytic bacteria in abiotic stress management (Malfanova *et al*., 2011; Hashem *et al*., 2016). Endophytic bacteria have been reported in different crop plants, aromatic and medicinal plants, halophytes etc. (Guimarães *et al*., 2012; Sharma *et al*., 2012; Egamberdieva *et al*., 2017). The beneficial associations of root colonized microbes and plants bring about increased uptake of nutrients, and altered metabolic developments in the plants. The endophytic bacteria directly or indirectly mitigate the harmful imparts of salt stress in plants (Hashem *et al*., 2016). The ability to produce plethora of secondary metabolites, plant growth regulators, (Table 2.3) are the characteristic bioactive molecules involved in the of alleviation of

salinity stress in plants by endophytic bacteria. (Beneduzi *et al*., 2012) However, despite the importance of the endophyte-plant relationship, our knowledge on the interactions between legumes, endophytes, and pathogens under hostile environmental conditions is yet to be explored. Medicinal weeds have also been reported as a rich source for the isolation of endophytic bacteria (Berg *et al*., 2013).

2.7 Mode of entry of bacterial endophytes into plant system:

Bacterial endophytes employ different mechanisms to gain entry into the plant tissues; excluding the seed endophytes, the most usual mode of entry of endophytes is through primary and lateral root cracks (Agarwal and Shinde, 1987; Sprent and de Faria,1998; Sørensen and Sessitsch, 2015).The leakage of metabolites from the wounded regions of the plant, serves as stimulants for bacterial entry and colonization (Hallmann *et al*., 1997).Sometimes, bacteria can also enter the plant through natural openings such as stomata on young leaves, (Roos and Hattingh, 1983); lenticels on stem (Scott *et al*., 1996); or germinating radicles (Gagné *et al.,*1987).They can also make an entry via emergence of lateral roots or root hair cells(Huang, 1986). Some of the studies reveal some other mode of entry of endophytes into plants, for example, the entry of endophytic bacterium *Enterobacter asburiae* JM22 in cotton plants was assisted by the bacterial cellulase enzyme (Hallmann *et al*.1997). In another case, the bacterial species *Herbaspirillum seropedicae*, which shows another mechanism than the usual known mechanisms because it lacks genes for degrading plant cell walls, but it is also a successful endophyte (Pedrosa *et al*., 2011; Wisniewski-Dyé *et al*.,2011). It is still not clear about the actual mechanism behind the entry of bacteria into the plant system and their interaction among themselves. (Spaink, 2000).

2.8 Mechanisms employed by bacterial Endophytes in inducing salt stress tolerance in crop plants:

Endophytic bacteria employ few molecular mechanisms such as production of certain specific bioactive molecules including phyto hormones, certain osmoprotectants, ACC deaminase, metal chelating compounds such as siderophores, some antioxidants. These biomolecules synthesized by the bacteria while residing inside the plant tissues, not only help them to survive in stress conditions but also help in plant stress response enabling the growth and development of plants in saline environment

6.1. Secretion of phytohormones

The phytohormones which play a key role in plant growth promotion include auxins such as IAA, Gibberlic acid (GA), Cytokinins etc bacterial endophytes are well known in production of these hormones and beneficial effect is directly observed in the plants. There are many reports on bacterial modulation of phytohormones production in crop plants. Radish seeds pretreated with *Bacillus subtilis* and *Pseudomonas fluorescens* elevate IAA and GA content in radish and increase its stress tolerance against soil salinity (Mohamed and Gomaa, 2012).IAA has key role in seed germination and root cell elongation (Zimmer *et al*., 1995).Increased levels of IAA in plants due to bacterial endophytes decreases ill effects of salt stress such as stomatal closure, cell wall extrusion. etc.(Ribaut and Pilet, 1994).Bacterial endophytes increase the endogenous IAA synthesis and compensate for salt induced reduction of IAA in plants (Liu *et al*.,2013).Another hormone Abscisic acid (ABA) has a deleterious effect on plants during salt stress conditions such as inhibition of shoot and leaf growth, closure of stomata (Kang *et al*.,2014).The bacterial endophytes ameliorate these effects caused by ABA by stimulating the stress responsive genes and inhibiting the ABA synthesis (Herrera-Medina *et al*.,2007). Salicylic acid plays an important role in Scavenging reactive oxygen species (ROS) and induces systemic tolerance to the salt stress in plants (Radhakrishnan and Lee, 2013).Some of the endophytic bacteria such as *Enterobacter sp*. SE992 and *Pseudomonas putida* H-2-3 under saline conditions increase SA accumulation in plants and thus improve plant growth (Kang *et al*., 2014, 2015).

2.8.2 Synthesis of antioxidants

During salinity stress, the plants generate reactive oxygen species(ROS) such as singlet oxygen, hydrogen peroxide, superoxide radical, and hydroxyl radical mitochondria, peroxisomes, plasma membrane, endoplasmic reticulum and cell wall (Choudhury *et al*., 2013).Accumulation of the ROS is a highly damaging phenomenon which damages bio molecules such as, the lipids, proteins and nucleic acids (Yokoi and Rengel, 2002).It has got cascading effects such as increasing the rate of lipid peroxidation, decreasing the rate of scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbic peroxidase (APX) in salt-affected plants(Halo *et al*., 2015). Malondialdehyde (MDA) accumulation is one of the response of the plants to salinity stress which disturbs the structural integrity of cellular membranes while bacterial inoculation suppresses the

toxic effects caused by MDA accumulation in plants. (Wu *et al*., 2014).During salinity stress, the protease enzyme degrades proteins in cells under stress conditions and similar to ROS, a caspase-like activity is involved in programmed cell death (Keyster *et al*., 2012). Cell caspase-like protease activity is triggered in plants during salinity, but *Pseudomonas alcaligenes* and *Bacillus pumulis* decrease the salinity induced elevation of protease activity, thus helping the plants to survive in stress conditions (Jha and Subramanian, 2014). Catalase (CAT), Superoxide dismutase (SOD), and Peroxidase (POD) enzymes are considered as major ROS-scavenging enzymes in plants. CAT decomposes hydrogen peroxide to water and oxygen. Similarly, SOD converts superoxide free radical into oxygen and hydrogen peroxide (H_2O_2). POD and some other enzymes are involved in the degradation of H_2O_2 into harmless products. An increased activity of antioxidants can be considered as one of mechanisms involved in salt stress tolerance in plants (Hernandez *et al*.,2003). Bacterial inoculation in salt-affected soil shows a higher activity of ROS scavenging enzymes in plants, which is indicative of the mitigation of salt stress (Bianco and Defez, 2009).

2.8.3 Secretion of osmolytes

Production of osmolytes is considered as one of the important mechanisms conferring plants tolerance against salinity stress. Osmolytes such as proline, lysine and sugar are well known osmoprotectants that maintain cell turgor (Weinberg *et al*., 1982; Mundree *et al*., 2002) to prevent oxidative stress. Proline content is increased during the salt stress in plants and it stabilizes proteins and cell membranes and also scavenge ROS (Claussen, 2005). Some of the other osmolytes such as, glycine, betaine (GB) and quaternary ammonium compounds (QAC) are not produced usually in normal conditions in rice, mustard, and Arabidopsis in natural, but their concentration is remarkably increased during salt stress conditions (Rhodes and Hanson, 1993). In addition, osmolytes also maintain the oxygen-evolving activity of photosystem-II protein complex by protecting against the dissociation of regulatory extrinsic proteins and manganese cluster. An increase of GB-QAC in stressed plants plays a key role in enhancing the Na-flux from cytoplasm to vacuole and regulates membrane function (Yeo, 1998). Moreover, sugars (glucose, fructose, trehalose, and mannitol) accumulation in plants under salt stress condition prevents structural and functional changes in membranes and proteins (Ashraf *et al*., 1991; El-Haddad and O'Leary, 1994). During bacterial interaction with salt-affected

plants, different methods have been used to stimulate compatible solutes for enhancing plant stress tolerance. For example, *Streptomyces sp*.PGPA39 inoculation suppresses the proline accumulation in salt injured tomato plants (Palaniyandi *et al*., 2014). *Bacillus amyloliquefaciens* NBRISN13 induces more synthesis of proline in rice than in salt-stressed plants (Nautiyal *et al*., 2013). *Pseudomonas pseudo alcaligenes* and *Bacillus pumilus* reduce proline content and increase the GB-QAC in rice plants to mitigate the salinity stress effects (Jha *et al*., 2011). Proline and QACs possibly induce development of strong H-bonded water around the protein to preserve the native state of the cell biopolymers (Kumar *et al*., 2003).However, bacteria reduce the total sugar content in salt-stressed wheat plants (Nabti *et al*., 2010). Some of the bacteria promote the synthesis of proline, GB, choline, and total soluble sugar in chickpea plants to facilitate the plants in adapting to the salt stress conditions (Upadhyay and Singh 2015; Qurashi and Sabri 2013).

2.8.4 Synthesis of ACC deaminase

Ethylene, is an important the plant growth hormone that under normal conditions(in low concentration induces plant growth, but under abiotic stress its concentration increases and exerts negative on plants development process (Kang et al., 2014).It has got both positive and negative effectson plant developmental process. The exogenous application of ethylene or its direct precursor 1-aminocyclopropane-1-carboxylate (ACC) significantly inhibited the elongation of infection thread and formation of nodules in most of the legumes.(Glick 2015) Bacterial endophytes are excellent candidates in mitigating the ill effects of ethylene accumulation in the plants through production of the enzyme ACC deaminase. One of the direct evidence of involvement of ACC deaminase in endophytic plant growth promotion comes from *Burkholderia phytofirmans* PsJN in canola seedlings. (Sessitsch *et al*., 2005).Further evaluation of the same with the mutant bacterium showed no growth promotion in canola seedlings, owing to the fact that it lost the ACC deaminase activity(Sun *et al*., 2009).The Other reports on potentiality of endophytic bacteria in ACC deaminase production include mitigation of salt stress in *Catharanthus roseus* (Karthikeyan *et al*., 2012), osmotic stress in pepper plants (Sziderics *et al*., 2007) and copper stress in canola (Zhang *et al*.,2011).The cross talk between bacterial ethylene production and plant ethylene production is very interesting and it involves the syngerstic action of various harmones such as IAA in the regulation of plant ethylene levels under abiotic conditions as shown in the fig.2.4

Table 2.3. Plant- endophytic bacterial interactions and the physiological changes in plants under salt stress.:

Bacteria	Bacterial seceretions	NaCl/EC ranges	Plant functional studies	References
Achromobacter piechaudii ARV8	ACC deaminase	0-207 mM	Tomato plant growth Water use efficiency, osmotic concentration, ethylene, Ca, Mg, K, Na, P, S, Ba and Fe	Mayak *et al*., 2004
Ochrobactrum intermedium L115	Organic acids, IAA, siderophore and ACC deaminase	0-300 mM	Peanut plant growth Fatty acids and phosphatidylcholine	Paulucci *et al*., 2015
Oceanobacillus profundus Pmt2	Exopolysaccharides	0-200 mM	*Lens esculenta* plant growth Proline, glycine betaine and photosynthetic pigments	Qurashi and Sabri 2011
Micrococcus yunnanensis PGPB7	IAA, phosphate solubilization and siderophore production	0-200 mM	*Arabidopsis* and rice plant growth Salinity responsive genes (AtRSA1, AtVQ9 andAtWRKY8)	Sukweenadhi *et al*., 2015

Klebsiella oxytoca Rs-5	IAA and ACC deaminase	0-120 mM	Cotton seed germination, plant growth MDA, proline and IAA	Liu *et al*., 2013
Halomonas sp. SL 9	IAA, siderophore production and ammonia Production	0e4.6 ds/m	Wheat plant growth Photosynthetic pigments, protein, flavonoids, proline, sugar and phenolic acids	Tiwari *et al*., 2011
Haererohalobacter strain JG-11		0-100 mM	Peanut plant growth Water content, MDA, Ca, K, Na, P, N, proline, sugar, amino acids, protein and auxin	Shukla *et al*., 2012
Geobacillus caldoxylosilyticus		0-350 mM	Maize plant growth Water content, Na, Cl, K, proline	Abdelkader and Esawy

IRD			photosynthetic pigments, CAT and SOD	2011
Exiguobacterium oxidotolerans STR36	Phosphate solubilization and exopolysaccharide production	0-500 mM	Mentha arvensis plant growth Photosynthetic pigments, proline, MDA, CAT, APX, oil content, essential oil composition, Na, K and P	Bharti *et al.,* 2014
Enterobacter cloacae W6	ACC deaminase	0e14.2 ds/m	Wheat seed germination, plant growth and yield Na, N, P and K	Nadeem *et al*., 2013
Curtobacterium flaccumfaciens E108	IAA and phosphate solubilization	4.8%	Barley seed germination, biomass and water transport Na, Ca, K and Mg	Cardinale *et al.,* 2015

Bacillus amyloliquefaciens NBRISN13	IAA, phosphate solubilization and ACC deaminase	0-200 mM	Rice plant growth Chlorophyll, proline, expression patterns of various salt (NHX1, SOS1, BZ8, GIG, BADH, SAPK4 and SNRK2), defense (LYSO, CAT, PBZ1 and SERK1) and diverse stress responsive (MAPK5, NADP-Me2 and EREBP) genes	Nautiyal *et al.*, 2013
Azotobacter sp. C5	IAA, phosphate solubilization and nitrogen fixation	0e5.85 g/ Kg soil	Maize plant growth Chlorophyll, Ng, K, Ca, Mg, total polyphenol and proline	Rojas-Tapias *et al*., 2012
Arthrobacter sp. AS 18	IAA, Siderophore production, ACC deaminase and proline	0e4.6 ds/m	Wheat plant growth Photosynthetic pigments, protein, flavonoids,	Tiwari *et al*., 2011

	accumulation		proline, sugar and phenolic acids	
Alcaligens sp. SB1-ACC2	ACC deaminase, IAA, siderophore, solubilization of phosphate and ammonia production	0-150 mM	Rice seed germination and plant growth Chlorophyll	Bal *et al*., 2013
Aeromonas hydrophila/caviae MAS-765	Exopolysaccharide	0e8.0 ds/M	Wheat plant growth K, Na and Ca	Ashraf *et al.,* 2004
Acinetobacter calcoaceticus SE370	Gibberellins, phosphate solubilization	0-120 mM	Cucumber plant growth Water content, chlorophyll, ABA, SA, GA, CAT, PPO, POD, Polyphenol, Na, K and P	Kang *et al.,* 2014

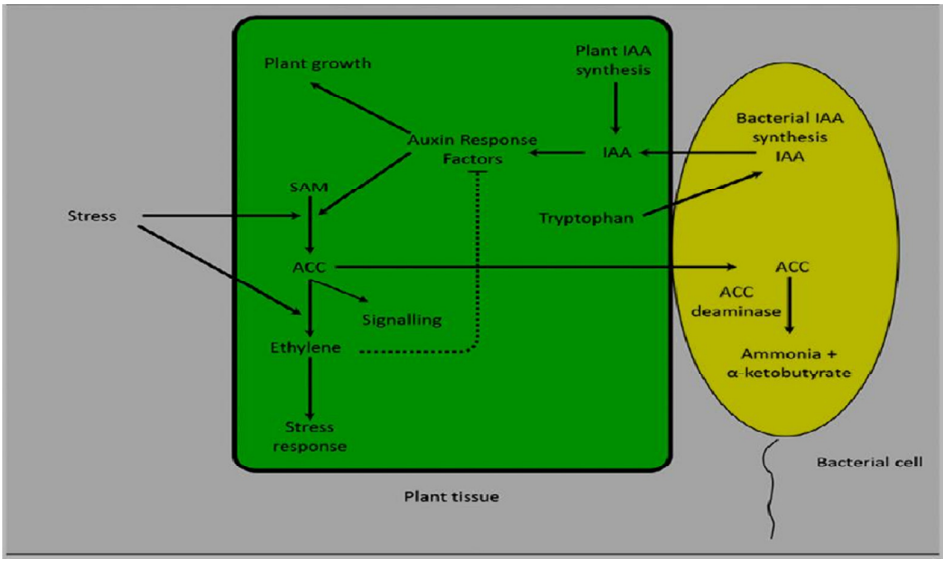


Fig.2.4.Schematic representation of mitigation of ethylene accumulation in plants during abiotic stress through ACC deaminase activity of bacterial endophytes (**Source:** Gamalero and Glick, Plant ethylene modulation by beneficial bacteria, 2015)

2.8.5 : Nutrient stress amelioration

Plant health ultimately depends on the supply and limitation of nutrients in the soil system. Availability of these nutrients depends on various factors such as soil pH, moisture, texture, and micro flora composition. As most of the nutrients are available in soluble forms at the soil pH range of 5–7. Salt stress changes the pH of soil by which most of compounds bound to cations and anions to form a stable compounds that makes them less available in soil. There insoluble fixed compounds are solubilized in soil by the enzymes/acids produced by PGPR and made available to plants.

(i) P- solublization:

One of the most important macro nutrients in plant growth promotion and development is Phosphorus (P) and it's 30-65 % present in organic form (30–65 %) in the soil which is not assimilated by plants. The organic P in soils is present in the form of inositol phosphatases, phosphoesters, phosphodiesters and phosphotriesters (Sindhu *et al*. 2010).But, the farming community undermines this concept and large amounts of phosphatic fertilizers are dumped into the fields leading to the serious environmental problems. Phosphate-solubilizing bacteria (PSB) that belong to the genera *Bacillus*, *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Brevibacterium*, *Corynebacterium*, *Serratia*

and *Xanthomonas* and fungal genera *Trichoderma*, are capable of hydrolyzing unavailable form of phosphorus in available form (Sindhu *et al*. 2010). These bacteria are known to secrete low molecular weight organic acids such as gluconic acid, citric acid, succinic acid, propionic acid, malic acid, oxalic acid and lactic acids that mineralize and dissolve organic phosphate compounds making them available to plants in the form of inorganic phosphate (Choudhary 2012). Some of the key enzymes involved in hydrolyzing the phosphate from organic soil are phosphatases and phytases. In addition to these, secretion of hydrogen ions in rhizosphere environments also alters the pH sufficiently to mobilize soil minerals (Khan *et al*. 2013).Salt stress in the soils causes depletion and precipitation of available phosphorous in the soil thereby reducing phosphorous avaliablity to crop plants, In this regard PSM, have tendency to solubilize precipitated forms of phosphorus in hydroponic MS medium and enhance the phosphorus content in plant system under NaCl stress (Shukla *et al*. 2012; Vaishnav *et al*. 2015). Bacterial-inoculated plants exhibit higher phosphatase activity which released soluble phosphate from its insoluble compounds inside the plant cells and helped plants to tolerate salt stress Kohler *et al*. (2008).

ii) Enhancing nitrogen availability:

Another central element in plant nutrition is nitrogen it is an essential component required for protein and nucleic acid synthesis. Certain soil and endophytic microorganisms have the capacity to fix atmospheric nitrogen (N_2) and provide it to plants in the form of ammonia via nitrogen fixation process. Symbiotic nitrogen fixation accounts for nearly 65 % of the total biologically fixed nitrogen (Rajwar *et al*. 2013). Adverse effects of salinity on legume-rhizobium symbiosis, including the deleterious effect on diversity of rhizobia in soil, interaction between legumes, reduction in the number of nodules. In the past few years, salt-tolerant PGPR which can tolerate higher levels of salts, up to 1.5–2.0 M NaCl, were co-inoculated with *Rhizobium* in legumes for growth enhancement and successful N_2 fixation (Divito and Sadras, 2014). The co-inoculation of PGPR is a good strategy when *Rhizobium* is not so effective in saline environment. ACC-deaminase-containing PGPR have been observed to reduce the ethylene concentration which decreases nodulation efficiency in legumes under stress environment (Ahmad *et al*. 2011). *Pseudomonas* and *Rhizobium phaseoli* co-inoculation was observed very effective for enhancing nodulation process in mung bean plants under salt stress conditions grown in laboratory as well as in fields. (Ahmad *et al*. 2013). Similarly, co-inoculation of

Mesorhizobium sp. with IAA-producing *Pseudomonas* has been found to increase nodulation in chickpea (Malik and Sindhu, 2011). In another study, co-inoculation of *Pseudomonas* and *Rhizobium* was found to increase nodulation and nutrient uptake (Mishra *et al*. 2011). Furthermore, coculture of *Azospirillum* and *Rhizobium* was found to enhance nodulation which alsoincreased tolerance in plants against unfavourable conditions (Bashan and de-Bashan 2010).

iii) Enhancing iron availablity

Iron is the fourth most abundant element(micronutrient) required by most of the living organisms for growth. It plays a key role as cofactor for nearly 140 enzymes catalyzing specific biochemical reactions and processes. Under oxic conditions iron, exists in the ferric state ($Fe3^{+}$) and in aqueous phase produces insoluble hydroxides and oxyhydroxides which are not readily available to plants and microorganisms (Ma *et al*. 2011). In saline soil, the availability of iron is further reduced due to decreased solubility from lower pH to higher pH (Thomine and Lanquar, 2011). Organisms have employed various mechanisms to get available form of iron; among them siderophores have been best studied. Siderophores are iron-chelating agents and proved in different PGPR strains as an important attribute for plant growth promotion and phytopathogen protection (Scavino and Pedraza 2013). PGPR secrete siderophore in the rhizosphere, where they chelate iron and then plant roots uptake iron from siderophore by either chelate degradation or direct uptake (Rajkumar *et al*. 2010). Siderophores have been concerned for both direct and indirect mechanisms of plant growth promotion by PGPR. Sharma and Johri (2003) reported that siderophore-producing *Pseudomonas* spp. strains GRP3A and PRS significantly increased maize seed germination and plant growth under iron-stressed condition and suggested application of these bacterial strains for crop productivity in calcareous soil system.

iv) Enhancing potassium availability:

Third most essential macro nutrient involved in plant growth and development is potassium (K) which is involved in various metabolic processes in plants (Sindhu *et al*. 2010). Potassium is present in soil in both available (water soluble) and unavailable (micas, illite and orthoclase)forms. The common components of potassium in the soil are feldspar and mica in 90–98 % (Sindhu *et al.* 2010).Potassium-solubilizing bacteria (KSB)

are able to release K from its unavailable form. Meena *et al.* (2014) described the importance of KSB in K uptake efficiency of by plants and reduction in the use of costly chemical fertilizers. Three PGPR strains (*B. mucilaginosus*, *Azotobacter chroococcum* and *Rhizobium* sp.) were found to solubilize K from waste mica and enhance its uptake in maize and wheat plants under salt stress conditions (Singh *et al*. 2010).

v) Enhancing sulphur availability:

Sulphur is also one of the essential nutrients but only a fraction of the essential nutrients, but only a fraction of total soil sulfur, i.e, 5% is available for plants as so_4^{-2} , while 95% of sulfur is found in bound form as pyrite(FeS_2) ,gypsum ($CaSO_4.2H_2O$) and epsonite ($MgSO_4.7H_2O$), which are unavailable for plants. This unavailable sulphur is made available to plants where soil microbiota plays an important role by either by biochemical or biological mineralization(Gharmakher *et al*. 2009) Salt stress affects the availability of sulphur to the plants forming undesirable complexes. Sulphur-oxidizing bacteria are chemoautotrophic and photosynthetic bacteria which include *Beggiatoa*, *Chromatium*, *Chlorobium*, *Thiobacillus*, *Sulfolobus*, *Thiospira* and *Thiomicrospira*. Common PGPR species such as *Bacillus* and *Pseudomonas* have been reported to reduce sulphate to H_2S (Sindhu *et al*. 2010).

vi) Enhancing Zinc availability:

Zinc is also one of the important micro nutrients which is deficient in most of our agricultural soils. It plays several functions throughout the life of plants. It is also major element involved in many redox reactions and performs many important functions in plants such as auxin synthesis, photochemical reactions of chlorophyll, stability of biological membranes and SOD and carbonic anhydrase enzymatic activity (Broadley *et al*. 2007). Zinc nutrition is critical for plant growth, maturity, seed quality and yield. Zinc is present in the soil as ZnS (sphalerite), and mineral ores such as smithsonite ($ZnCO_3$), zincite (ZnO), zinkosite ($ZnSO_4$), franklinite ($ZnFe_2O_4$) and hopeite [Zn_3 $(PO_4)_2$ $4H_2O$]. Tariq *et al*. (2007) have found that Zn-mobilizing bacteria enhanced Zn uptake in rice seedlings which had positive impact on plant growth and grain yield. In another study, *Serratia* sp. has been noted to solubilize ZnO and was able to significantly increased wheat yield under salt stress conditions (Abaid-Ullah *et al*. 2015).

2.9 Expression of salt responsive genes/proteins in conferring salt tolerance by bacterial endophytes under stress conditions:

Salinity stress induces several in morphological, physiological and molecular changes in plant and in turn plants also respond to these changes by modifying the expression of genes under those conditions. In rice plants, inoculation with endophytic *Bacillus amyloliquefaciens* NBRISN13 up regulates the SERK1 genes (somatic embryogenesis receptor-like kinase) and suppresses the ethylene responsive element binding proteins (EREBP)expressing genes. Salt stress reduces the expression of salt-responsive genes such as those encoding sodium proton antiporter (NHX1), salt-overly-sensitive 1 (SOS1), gigantean (GIG), betaine, aldehyde dehydrogenase (BADH), serine-threonine protein kinase (SAPK4), and sucrose nonfermenting-1-related protein kinase 2 proteins (SNRK2); bacterial endophytic colonization and endophytic bacteria are known to mitigate this by up regulation of these genes (Nautiyal *et al*., 2013).Salt stress also downregulates the expression of defense genes such as, those encoding for lysophospholipase (LYSO), CAT, probenazole responsive homologous to PR 10 (pBZ1), and somatic embryogenesis receptor-like kinase 1(SERK1) but the endophytic bacterial inoculation upregulates the expression of these genes and confers the tolerance to plants (Nautiyal *et al*., 2013).In Arabidopsis plants, *Burkholderia phytofirmans* PsJN inoculation accelerates the expression of ROS scavenging (APX2), and detoxification genes and also regulates ROS, ABA, and JA biosynthesis (Pinedo *et al*., 2015).They also increase the expression of antioxidant genes such as DHAR (dehydroascorbate reductase), GR, APX, and CAT in okra plants(Habib *et al*., 2016).

2.10 Successful instances of bacterial endophyte mediated alleviation of salt stress:

Endophytic bacteria are better equipped than the rhizospheric bacteria in inducing strong defense responses against stresses in plants (Andrews, 1992, Pandey *et al*.,2012). There are many reports on endophytic bacteria mediated mitigation of abiotic strees. Jha *et al*.,(2011) have reported that when an endophytic bacterium *Pseudomonas pseudoalcaligenes* combined with a rhizospheric *Bacillus pumilus* was inoculated in paddy, it protected paddy plants from abiotic stress by induction of osmoprotectant and antioxidant proteins. Endophytic bacteria *P. pseudoalcaligenes* at lower concentrations of salinity levels showed a considerable concentration of glycine betaine-like quaternary compounds production and increase in shoot biomass. While co inoculation of both *P.*

pseudoalcaligenes and *B. pumilus* at higher salinity levels showed improved response against the adverse effects of salinity. Production of exopolysaccharides is known as one of the important mechanism in conferring salt stress tolerance to wheat plants under high salt stress conditions (Grover *et al*.,2010). An endophyte from wheat, *Pseudomonas aeruginosa* PW09 was evaluated for its ability to alleviate abiotic stress in cucumber. (Pandey *et al*.,2012). Generally stresses have lethal effects on plant development and growth. Bacterial endophyte inoculation may recover plant health in stress conditions.

Materials and Methods

3.1 Isolation of bacterial endophytes

Endophytic bacteria, were isolated from medicinal plants (*Phyllanthus* sp) following the method described by Mcinroy and kloepper, (1995). The plant samples were collected from wasteland infested with weeds at G.B.P.U.A& T, Pantnagar. The samples were washed under running tap water, root/stem portions were cut into 2-3 cm and pieces of fruits were separated. All the samples were surface sterilized using 70 % ethanol for 1 min, washed with sterile distilled water Then they were washed with sterile distill water to remove traces of ethanol. Further, they were treated by 0.1 % $Hgcl_2$ solution for 30 seconds followed by washing with 1% KCl solution thrice and drained. For isolation of endophytes, the surface sterilized plant samples were aseptically crushed using pestle mortar with few drops of sterile distill water in laminar air flow system. Once the plant samples were completely crushed into thick liquid paste, it was collected in sterile eppendr of vials. About 50 µl was spread using sterile glass spreader on trypic soya agar and also streaked on the TSA plates. The medium was supplemented with Cyclohexamide (50 ppm) to avoid fungal contamination. The inoculated plates were incubated at $28^0 \pm 2^0$ for 2 days. The isolated distinct colonies were re-streaked on fresh medium. The purified cultures were preserved after proper indexing

3.2 Composition of culture media used

i) Trypticase soy Agar (TSA) medium (gL^{-1})

Components:	Concentrations (gL^{-1})
1.Pancreatic digest of casein	15.0
2.Peptic digest of soybean meal	5.0
3.Sodium chloride	15.0
4. Agar	15.0
pH	7.0
Distill water	1000 ml

ii) Nutrient agar

Components:	Concentration (gL^{-1})
1. Yeast extract	3.0
2. Peptone	5.0
3. Sodium chloride	5.0
4. Agar	15.0
pH	7.0± 0.2
Distill water	1000 ml

3.3 Maintenance and preservation of culture

All the isolated and purified endophytic bacterial cultures were maintained and preserved on NA slants at 4^0 c in refrigerator.

3.4 *In vitro* screening of cultures for abiotic stress (pH and salt) tolerance: Plate assay

Twenty seven endophytic bacteria isolated from different plant parts, were screened in vitro for their alkali limits and salt tolerance limits. For the determining the pH tolerance limits, the bacteria were grown on nutrient agar media amended with different pH buffer solutions (pH 7.0-11.0).The alkalo tolerant bacteria obtained from screening process(11), were further screened for salt tolerance limits by growing them on NA amended with salt concentration ranging from 0 to 12%.The selected isolates were inoculated on the agar plates in a checker board arrangement and the plates were visualized for the growth of bacterial isolates.

3.5 Qualitative screening of bacterial endophytes for Plant growth promoting traits

All the selected eleven cultures showing higher pH and salt tolerance were evaluated qualitatively for plant growth promoting traits, the cultures were revived a active cultures (O.D. 0.8) were used for experiments

3.5.1 Phosphate (P) - solublization assay

A preliminary experiment was performed to assess the potential of isolated endophytic bacterial cultures using in soluble forms of Phosphorus such as, tricalcium phosphate as a phosphate source amended in mineral salt medium(gL^{-1})Glucose:10.0, TCP:1.0, $Mgcl_2$:4.15, NH_4No_3:2.0, KCL: 0.3, $MgSo_4$: 4.0 gm/l, distill water make up the volume 1000 ml. Active bacterial cultures(O.D. 0.8) were point inoculated on TCP amended plates with the help of sterile tooth picks, and incubated at 28 ^{0} c ± 2 ^{0} c. The P-solubilizing abilities of different cultures were assessed by the appearance of zone of clearance around the colonies. **(Nautiyal CS., 1998)**

3.5.2 IAA production

In order to assess the IAA production ability of all eleven selected bacterial endophytes, a test was performed using nutrient Broth (10 ml) supplemented with 0.5 g% tryptophan was inoculated with 0.1 ml [1% (v/v)] inoculum. It was then incubated for 48 h in a rotary shaker incubator, set at 30^o± 2 ^{0} c with a shaking speed of 120 rpm. After incubation, the broth culture was centrifuged at 10,000 rpm for 15 minutes at 4 ^{o}C. To 1 ml of supernatant, 2ml Salkowski reagent (50 ml of 35% perchloric acid, 1 ml of 0.5 M $FeCl_3.6H_2O$ solution) was added. It was incubated in the dark for 30 minutes at room temperature (37 ^{0} c) Uninoculated sterile medium served as the control. Development of cherry red colour indicated IAA production by the endophytic bacteria. **(Bhardwaj *et al.*, 2008)**

3.5.3 Siderophore production

All the selected bacterial endophytes were tested qualitatively for *in vitro* siderophore producing efficacy by (CAS) agar plate assay**.** The active bacterial cultures were spot inoculated and in the centre of CAS agar plates (pH 7.0) and incubated at 30±1ºC for 5d, appearance of yellow/orange zone around the colonies indicated siderophore production test **(Schwyn and Neilands, 1987)**

3.5.4 HCN production

All the selected bacterial isolates were streaked on nutrient agar (NA) medium supplemented with 4.4 gL-1 of glycine. The production of cyanide was detected by placing Whatman filter paper No.1 soaked in 0.5% picric acid under side of the petri dish lids.

Development of brown to red color after incubation of four days indicated HCN production ((**Bakker and Schipper, 1987**)

3.5.5 Urease production

For determining urease producing abilities of the test cultures, stuart's urea broth was prepared gL^{-1}(Yeast extract:0.1,Potassium phosphate, monobasic 9.1, potassium phosphate dibasic 9.5,Urea 20, phenol red 0.01).All these ingredients were dissolved in 1000 ml of distill water and filter sterilized(0.45-mm pore size) and 3 ml of broth is distributed to each tube inoculated with selected cultures and incubated for 24-48 hrs at 37 ºC, in which the appearance of red colour or cerise colour indicates the positive result for urease test. Yellow color indicates the negative result (Benita brink, 2010)

3.5.6 Ammonia production

Bacterial endophytes were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in peptone broth (10 ml) in each tube and incubated at28 ± 2°C for 48–72. Nessler's reagent was added to each tube after incubation. The development of faint yellow to dark brown color was a positive test for ammonia production (**Cappuccino and Sherman, 1992**)

3.6 Determination of growth curve of selected cultures under pH and salt stress conditions

The four bacterial cultures, selected on the basis of their pH and salt tolerance and *in vitro* ability to grow under abiotic stress quantitavely and growth curves were prepared.

The four selected bacterial isolates (PSBeP-8, PSBeP-11, PRBeP-10, PRBeP-6) were screened quantitavely for their pH tolerance at the pH range of 7.0 to 10.0 in nutrient broth amended with different pH buffers. Active cultures (O.D-0.8) at the rate of 10 %v/v) were inoculated in sterilized media and incubated at 28 ± 2 ^{0} c. Samples were withdrawn periodically at definite time intervals.

Similarly the salt tolerance abilities of the cultures were quantitavely studied by growing the cultures in nutrient broth amended with different concentrations of the salt, NaCl, ranging from 0% to 14%(w/v).The media were inoculated with active(O.D.0.8) bacterial cultures and incubated at 28 ± 2 ^{0} c. Samples were withdrawn periodically at

different time intervals and optical densities were measured spectrophotometrically and growth curve were plotted.

3.7 : Quantitative estimation of P-solublization of selected cultures under abiotic stress conditions

Based on the qualitative screening for P- solublization and other plant growth promoting traits. The four selected halo-akalo tolerant bacteria were tested quantitavely for their P- solubilizing efficacies using TCP as sole phosphorus source. The mineral salt medium containing TCP as sole phosphorus source was adjusted with different pH range from 7.0 to 1.0 with appropriate buffer solutions. Similarly to determine P- solubilizing efficacies under salt stress were inoculated in the mineral salt medium amended with different concentrations of NaCl ranging from 0% to 12%.All the inoculated cultures were incubated in incubator shaker at 28 ± 2 ^{0} c(120 rpm) for 4d. After incubation samples were withdrawn periodically, centrifuged at 8000 rpm(0 ^{0} c) and culture filtrate/supernatant were estimated for available phosphate concentration.

3.7.1 Stock solutions and reagents used

a. 10mM stock solution of KH_2PO_4

b.2.5 % Ammonium molybdate solution- 2.5 g of ammonium molybdate ($(NH_4)Mo_7O_27.H_2O$) was dissolved in distilled water(20ml) and 10 N H_2SO_4 (50 ml) was added to it. The volume was made up to 100ml with distilled water.

c. Coloring reagent (10 ml)-

i. 15 % sodium bisulphide solution.

ii. 20 % sodium sulphide solution.

iii. 25 mg 1-amino-2-napthol 4-sulphonic acid (ANS).

250 µL of solution (ii) was added to the same volume of solution (i), then 25 mg ANS was added to it, mixed thoroughly and filtered through whatman No. 1 filter paper, final volume was made up to 10 mL with distilled water.

d. 60% perchloric acid (PCA) solution.

3.7.2 Standard curve

Distilled water (5ml) was taken as control. Aliquots of 50, 100, 150, 200, 250, 300, 350, 400, 450,450,500,550,600 and 650 µL (47.5, 95.0, 142.4……. and 475 µg of phosphate respectively) of KH_2PO_4 stock solution were taken separately in test tubes and volume was made up to 1ml by adding distilled water. Then, PCA solution(400 µl), was added to each tube, followed by addition of 400 µl of 2.5% of ammonium molybdate solution. Now, the coloring reagent, ANS (200 µL) was added to each tube and final volume was made upto 6.0 ml by adding distill water. The tubes were then incubated at room temperature for 15 mins. The absorbance was recorded at 640nm and standard curve (O.D V/s concentration) was drawn for phosphate estimation.

3.7.3 Determination of Phosphate concentration in culture broth

Active bacterial cultures of four selected isolates were inoculated and grown for 4 d in mineral salt medium amended with TCP as sole P- source as discussed in section 3.7.The samples were withdrawn periodically at a regular interval of 24h, centrifuged at 8,000 rpm (4ºC) and supernatant was used for analysis. To the Culture filtrate (1ml), PCA solution (400 µL) was added followed by addition of ammonium molybdate solution (400µL) and ANS coloring reagent (200 µL).The final volume was made up to 6mL with distilled water in each tube and incubated for 10 min at room temperature. The absorbance was recorded at 640nm and concentration of solubilized phosphate in culture filtrate was determined using standard curve.

3.8 Quantification of siderophore production under abiotic stress conditions

All the four selected bacterial cultures were evaluated for their in vitro siderophore production potential under abiotic(pH and salt) stress conditions. Siderophore production was checked in iron deficient broth cultures using mineral salt medium. Influence of pH on siderophore production was tested at varying pH values from pH 7.0to 10.0.Infleunce of salt stress on siderophore production was tested in same medium amended with varying salt (NaCl) concentrations 0% to 12%(w/v).Active bacterial cultures(0.D.0.8) were inoculated individually in flasks and incubated at 30± 2 ^{0} c (200 rpm) for 5 d. The samples were withdrawn periodically at an interval of 24 h and centrifuged at 8000 rpm(4^0c) for 10 min. Siderophore production was tested in which, the flasks containing medium inoculated with bacterial isolates, were centrifuged at 8000 rpmfor 5 min, and the culture filtrate

(0.5ml), was added to CAS dye(0.5 ml) and kept for 10 min and samples were analyzed spectrophotometrically at 630 nm and absorbance of sample(reference) containing 1ml of distill water and 0.5 ml of CAS dye was added to it served as control. **(Schwyn and Neiland, 1987).**Siderophore Production was estimated by CAS assay Siderophore production was estimated as per cent siderophore (sid) units and calculated using the formula given below:

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

Where, Ar- absorbance of reference (uninoculated CAS reagent); As- absorbance of sample at 630 nm.

Based on the concentration of phosphate released in culture filtrate upon P solublization and siderophore production (% sid units) two superior bacterial isolates (PSPBeP-8 and PRBeP-10) were selected for further experiments.

3.9 Assessment of bacterial endophytes on seed germination under normal and abiotic stress conditions

3.9.1 Sterilization of seeds

The seeds of fenugreek variety Rtm-1 were obtained from vegetable research centre, Pantnagar, the fast growing, short duration and leafy vegetable which is moderately tolerant to salinity was selected. The seeds were surface sterilized with 0.1% mercuric chloride for 30 s and rinsed several times with distilled water again followed by washing with 1% Kcl solution for 1 min and finally washed with distill water, the surface sterilized seeds were soaked overnight, prior to using for in vitro germination tests by plate assay. Hartmann and Kester (1964).

3.9.2 Preparation of bacterial inocula

Two selected bacterial isolates PSBeP-8 and PRBep-10 were inoculated in the fresh nutrient broth flasks and incubated at 28 ± 2 ^{0} c for 24 h and (O.D 0.8) adjusted cultures were centrifuged at 8000 rpm at 4 ^{0}C for 10 min. The culture supernatants were discarded

and pellets were washed and re-suspended in sterile distilled water (SDW) to get final bacterial population density of 10^8 cells/ml. Seeds of fenugreek (cv Pusa early Bunching) coated with 1% CMC slurry without bacterial coating served as control. Fernando E. *et al*., 2000

3.9.3 Bacterial inoculation of seeds

The seeds were treated separately with the bacterial inocula, prepared according to the method described in 3.9.2. in which the some seeds were treated with isolate PRBeP-10,PSBeP-8 separately, they were also treated with 1% CMC slurry and incubated for 6-8 hours at 28 ± 2 ^{0} c for proper infection of seeds. After incubation, the seeds were dried inside the laminar air flow aseptically.

3.9.4 Seed viability test: plate assay

In order to evaluate the germination percentage of the fenugreek seeds, an experiment was performed , in which the towel paper was used as substrate on which the seeds were placed in petri plates, the towel paper was moistened with test solutions(Normal sterile distill water, pH solutions, salt solution) according to different experiments. The treated seeds were placed inside the petri plates which contains moistened towel paper with test solutions and lids of the petri plates were closed and kept for incubation at 28 ± 2 ^{0} c for 3 d and germination was checked for every 24 h interval.

a. Under normal conditions-paper towel with distill water

Properly sterilized seeds of fenugreek as discussed in 3.9.1.Were used for viability test by plate assay. 25 seeds were placed inside the petri plates containing towel paper, which was kept moistened with normal sterile distill water throughout the experiment.

b. Under pH stress conditions

The seeds inoculated with selected cultures(PRBeP-10,PSBeP-8) and uninoculated seeds were placed in the petri plates containing moist towel paper(with different pH solutions 7-10).25 seeds were placed in 3 replicates for each treatment and they were incubated at 28 ± 2 ^{0} c for 3 d and germination percentage was checked every 24 h interval.

c. Under salt stress conditions

The inoculated seeds were placed in petri plates containing towel papers moistened with different salt solutions ranging from(50-350 mM) of salt (NaCl) concentration and petri plates were incubated at 28 ± 2 ^{0} c for 3 d and germination percentage was checked every 24 h interval. (ISTA., 1993)

Layout of viability test:

S.No	Condition	Inoculation with bacterial endophytes	Treatment	Replications
1	Normal			
	pH-7	Uninoculated	T_0	3
		Inoculated with PRBeP-10	T_1	3
		Inoculated with PSBeP-8	T_3	3
	Salt concentration (0%)	Uninoculated	T4	3
		Inoculated with PRBeP-10	T5	3
		Inoculated with PSBeP-8	T6	3
2	Alkaline pH (8-10)	Uninoculated	T7	3
		Inoculated with PRBeP-10	T8	3
		Inoculated with PSBeP-8	T9	3
3	Saline conditions(50-350mM, NaCl, w/v)	Uninoculated	T10	3
		Inoculated with PRBeP-10	T11	3
		Inoculated with PSBeP-8	T12	3

3.10. Pot culture studies for evaluation of bacterial inoculation on performance of leafy vegetable fenugreek under abiotic stress conditions

The endophytic bacterial isolates (PSBeP-8, PRBeP-10 and co-culture of PSBeP-8 + PRBeP-10) were used for pot culture experiment conducted to evaluate the influence of bacterial inoculation on growth and leafy biomass yield of fenugreek under glass house conditions.

3.10.1 Collection and characterization of soil sample:

The saline soil used for pot experiment was collected from local salt affected field of shahjahanpur, Uttar Pradesh, India, the area well known for the widely spread salinity in north India. The soil samples were collected in polythene bags and brought to laboratory for analysis of various chemical characteristics such as pH, Electrical conductivity, available N,P,K. and some other important salts/ions.

3.10.2 Genotypic characters of fenugreek variety used:

The early maturing variety of fenugreek(Rtm-1/pusa early bunching) used in the present study used in the present study is pusa early bunching or Rtm-1, is a vigorously growing, semi erect medium sized variety, which is also moderately tolerant to salinity.

3.10.3 Development of bacterial inocula:

Selected endophytic bacterial mono cultures(PSBeP-8, PRBeP-10) and then co-cultures (PSBeP-8 + PRBeP-10) were grown in nutrient broth medium separately at 28 ± 2 ^{0} c for 24 h. All these cultures were centrifuged at 8000 g at 4^{0}C for 10 min. The culture supernatants were discarded and pellets were washed and re-suspended in sterile distilled water (SDW) to get final bacterial population density of 10^8 cells/ml. The cell suspensions of the two bacterial strains were mixed separately with 1% carboxymethylcellulose (CMC) solution in a ratio of 1:0.5 to form slurry coating onto the surface of sterilized seeds. **(Gupta *et al*., 2002)**

3.10.4 Sterilization and bacterial inoculation of seeds:

The seeds of fenugreek were surface sterilized with 0.1% mercuric chloride for 30 seconds followed by washing with 1% kcl solution and then washed several times by distill water. Prior to germination test the surface sterilized seeds were soaked in the sterile distill water for overnight. (Hartmann and Kester ,1964). The inocula of monocultures (PSBeP-8, PRBeP-10) and co-cultures (PSBeP-8 + PRBeP-10), containing cell density of 108 cells/ml were prepared as described in section 3.10.3, were mixed with 1% CMC solution in a ratio of 1:0.5 separately to form slurry for seed bacterization, the sterilized seeds were soaked in the bacterial slurry for 5 to 6 hours and were air dried for ½ h under sterile conditions under laminar air flow system **(Gupta *et al*., 2002)**

3.10.5 Experimental details/Treatments:

Experiment setup was layed out as completely randomized block design with different treatments under normal and abiotic The experiment was conducted using the soil at three levels of salinity:i. Non-saline(Normal soil) ii.Saline soil mixed with sand in the ratio 1:1(,50 % of saline soil) iii.Saline soil(100%).All the experiments were conducted as the three replicates as given in the table Saline soil in three replication for each, further details are given in table. The pots were arranged according to their treatments with respective replications

S.No	Soil conditions	Treatments	Replications
I	Normal soil		
	i.No treatment	T0	3
	ii.PSBeP-8	T1	3
	iii.PRBeP-10	T2	3
	iv. PSBeP-8 + PRBeP-10	T3	3
II	50% saline soil(saline soil:sand::1:1)		
	i.No treatment	T4	3
	ii.PSBeP-8	T5	3
	iii.PRBeP-10	T6	3
	iv. PSBeP-8 + PRBeP-10	T7	3
III	Saline/alkaline soil		
	i.No treatment	T8	3
	ii.PSBeP-8	T9	3
	iii.PRBeP-10	T10	3
	iv. PSBeP-8 + PRBeP-10	T11	3

3.10.6 Experimental setup:

The unsterilized soil samples were air dried in shade and grounded enough to make it homogenous and approximately 1.5 kg of the soil was put in each of the 2 kg plastic pot. The experiment was conducted using three sets of salinity conditions of the soil- Normal soil, 50% saline and 100% saline soil. Each set consisted of 4 levels of bacterial inoculation (seed coating) with monoculture of PSBeP-8, monoculture of PRBeP-10 and co-culture of PSBeP-8 + PRBeP-10, along with uninoculated control. Three replications were made for each treatment. Pots were watered prior to sowing of the seeds. The treated

and untreated seeds with selected cultures were sowed in the different pots(20 seeds per pot).The pots were regularly watered once in a day with sterile distill water and once in a week by Hoagland solution. After germination only 10 plants were maintained.

3.10.7 Observations

Various parameters related to vegetative growth and development aspects of the leafy vegetable (fenugreek) were taken such as, shoot length (cm), number of leaves per plant and fresh weight of stem were recorded at 15 and 30 days of germination (Wellburn,1994).

3.11 Characterization of potential bacterial endophytes:

The selected potential bacterial endophytes were characterized using phenotypic and molecular charcters.

3.11.1 Determination of morphological features of cultures

The cultures grown on the nutrient agar medium, were evaluated for various colony characteristics such as color, elevation, margin, texture, diameter etc.

The cultures were also visualized microscopically at 100 X for various cellular characteristics such as, cell size, cell shape, cell arrangement, gram reaction and sporulation.

3.11.2 Determination of Biochemical characteristics.

Both the endophytic bacterial isolates (PRBeP-10 and PSBeP-8) were tested for various biochemical traits, such as, catalase production, oxidase production, urease production and nitrate reduction which are considered as valuable criteria for differentiating and identifying various types of bacteria. These tests were carried out following the methods given in the laboratory manual (Cappuccino and Sherman)

3.11.3 Molecular characterization:

The selected endophytic bacterial isolates PRBeP-10 and culture PSBeP-8 were characterized on the basis of 16S r-RNA gene sequence analysis. Genomic DNA was extracted and PCR amplification of 16S rRNA gene was carried out using universal forward primer (5'-AGAGTRTGATCMTYGCTWAC-3') and reverse primer (3'-

CGYTAMCTTWTTACGRCT-5').The sequences 16SrRNA gene were then analyzed by BLAST at NCBI database. Phylogenetic tree based on 16SrRNA gene sequences was constructed by neighbour- joining plot method using MEGA7.

3.12. Statistical analysis.

All the experiments were performed in triplicate and data were expressed as average (Mean) values. Completely randomized design (CRD) with one or two factors was used. The experimental data was statistically analyzed by two way ANOVA using the statistical program STPR2 and means were compared. The statistical significance level (p-value) was set at 0.5 and results were compared using critical difference (CD) and standard error of mean(SEm).

Results and Discussion

Agricultural productivity heavily relies upon the soil health. Soil occupies an important place in sustainable agriculture. Anthropogenic causes coupled with natural calamities have caused a threat to soil health and productivity. Mismanagement of irrigation resources have resulted in conversion of fertile lands into saline or alkaline soils. In order to reclaim and rejuvenate degraded soils many strategies are being developed. Among them, microbial mediated approaches offer us eco-friendly and cost effective solutions for ameliorating the salt affected soils (Glick, 2007).Plant growth promoting endophytic microorganisms have largely remained as unexplored wealth, but recent studies suggest the importance and competence of these microorganisms in conferring stress tolerance to plants through their direct and indirect mechanisms such as, nitrogen fixation, siderophore production, phosphate solublization, phytohormones production and biocontrol activity, synthesis of antibiotics and volatile compounds production (Valencia-Cantero *et al*., 2007)

Main mechanisms by which endophytic bacteria confer abiotic stress tolerance to plants is still not clear. However, their occurrence inside the plant protect them from external abnormalities and in turn they are actively involved in regulation of plant ethylene levels, a major stress response hormone, osmolytes production, synthesis of antioxidants, maintaining ion homeostasis (Glick, 2012).Endophytic bacteria also increase the nutrient bioavailability in salt affected soils, as salt affected soils are deficient in many macro and micro nutrients which leads to poor crop establishment (Vardharajula *et al*., 2011). Underutilized medicinal weed plants are the treasure house of novel microorganisms known to possess the unique traits responsible for conferring medicinal properties to plants but, lack of intensive research in this area is still a surprising issue. These endophytic bacteria with the help of their various mechanisms confer necessary insulation against biotic and abiotic stresses. Salt stress not only confined to field crops but is also rapidly spreading and leafy vegetables are also affected severely due to this stress. One of the important leafy vegetables fenugreek (*Trigonella foeunum-graceum*), a moderately salt tolerant crop is severely affected by salt stress. Being a short duration crop, almost all growth stages of fenugreek are affected by salt stress.

In view of the above facts, present study was carried out to identify some novel potential endophytic bacteria from a medicinal weed plant *Phyllanthus* sp. *In vitro*/*In vivo* studies were conducted to explore the application of promising strains as bioinoculants for abiotic stress management of the plants grown under such soil conditions.

4.1 Bacterial endophytes from *Phyllanthus* sp

A total of 27 endophytic bacteria were isolated from different parts viz., root, stem and fruits of the freshly collected underutilized medicinal weed plant, *Phyllanthus* sp.(Table 4.1). The *Phyllanthus* sp. plants have been reported as a rich source of several secondary metabolites of pharmaceutical value and of endophytic micro flora. Many of the medicinal properties of this weed plant are attributed to the metabolic potential of these endophytic microorganisms residing inside the host plants. Interestingly, these plants are grown on waste and degraded lands naturally without any external addition of agro-chemicals. Therefore, an attempt is made in the present study to explore the beneficial micro flora(endophytic bacteria) from this weed plant, which can tolerate abiotic stress conditions and confer stress tolerance to inoculated plants. This study focused only on the isolation of endophytic bacteria from different plant parts of *phyllanthus* rather than other forms of microorganisms. There are several studies focusing the medicinal and pharmacological attributes of the Phyllanthus plants, but there only few studies with respect to endophytic microorganisms of this plant and their plant growth promoting abilities. Panchal and Ingal (2011) reported the occurrence of *Bacillus* sp as a major contributor to endophytic bacterial diversity in medicinal plant *chlorophytum borivilianum* (safed musli). Manoharan *et al*., (2016) reported the isolation of two salt tolerant endophytic and P-solublizing bacteria from *Phyllanthus amarus*. Apart from medicinal plants, the endophytic bacteria were also isolated other crops such as soybean (Li *et al*., 2008), grape vine (Trotel-Azize *et al*., 2008) and rice (Sang *et al*., 2014).

Table 4.1.Indexing of endophytic bacteria isolated from different parts of the medicinal weed plant *Phyllanthus s*p.

Sl.no	Site of collection of plant sample	Source	Plant part	Index of endophytic bacteria
1	Weed infested field,G.B.P.U.A&T, Pantnagar	*Phyllanthus* sp	Stem	i.PSBeP-1 ii. PSBeP-2 iii. PSBeP-3 iv.PSBeP-4 v.PSBeP-5 vi.PSBeP-6 vii.PSBeP-7 viii.PSBeP-8 ix. PSBeP-9 x.PSBeP-10 xi.PSBeP-11
2	Weed infested field,G.B.P.U.A&T, pantnagar	*Phyllanthus* sp	Fruit	xi. PFBeP- 1 xii. PFBeP-2 xiii. PFBeP-3 xiv.PFBeP-4 xv.PFBeP-5 xvi.PFBeP-6
3	Weed infested field,G.B.P.U.A&T, pantnagar	*Phyllanthus* sp	Root	xviii. PRBeP-1 xix.PRBeP-2 xx.PRBeP-3 xxi.PRBeP-4 xxii.PRBeP-5 xxiii.PRBeP-6 xxiv.PRBeP-7 xxv.PRBeP-8 xxvi.PRBeP-9 xxvii.PRBeP-10

4.2 Alkalotolerance of bacterial endophytes: Preliminary selection

Increasing alkalinity is becoming one of the serious problems as abiotic stress is limiting crop production worldwide. Therefore, *in vitro* study was conducted (Plate assay) was conducted to explore alkali tolerant limits of the bacterial endophytes. Out of twenty seven endophytic bacterial isolates tested for alkaline pH tolerance limits (pH7.0-10.0) only eleven isolates were able to grow up to alkaline pH 9.0 and only four endophytic

bacterial isolates test strains, PSBeP-8,PSBeP-11,PRBeP-6,PRBeP-10 exhibited growth up to pH 10.0 indicating their higher pH tolerance.(Table.4.2)

As per current literature available, normally bacteria can tolerate a soil reaction between pH levels 4.0 to10.5, but the most favorable pH for the majority of bacteria is just slightly alkaline side to neutrality (6.5-7.5).Endophytic bacteria are known to exhibit wide range of adaptability to diverse pH conditions, such as *Thiobacillus thioxidans* and *Acetobacter* sp are capable of growing at the very low pH (acidic) values pH range(pH 0-2) while, some *Bacillus* sp can grow at highly alkaline conditions with pH 11 (Berkeley and Campbelt,1972).Mechanisms that endophytic bacteria employ to survive under higher pH conditions are very diverse in nature, among them production of extracellular enzymes, increased acid production, changes in cell surface properties and increased in expression of ATP synthase are considered as possible mechanisms employed by them under alkaline stress conditions (Padan and shudiner,1994;Vijayalakshmi *et al*., 2011). Plate assay, as used by us during present investigation, has also been reported as an efficient method for preliminary selection of abiotic stress tolerant bacteria since it is based on their abilities to grow under such stress conditions(Madhumita *et al*., 2009).

Table 4.2.Preliminary selection of alkalo-tolerant endophytic bacterial isolates from *Phyllanthus* sp based on *In vitro* growth profile under alkaline conditions.

Sl.no	Bacterial Isolates	*In vitro* growth at different alkaline pH on agar plates				
		7.0	8.0	9.0	10.0	11.0
1	PSBeP-1	+	+	±	-	-
2	PSBeP-2	+	+	±	-	-
3	PSBeP-3	+	+	+	-	-
4	PSBeP-4	+	+	+	-	-
5	PSBeP-5	+	+	-	-	-
6	PSBeP-6	+	+	-	-	-
7	PSBeP-7	+	+	-	-	-
8	PSBeP-8	+	+	+	+	-
9	PSBeP-9	+	+	+	-	-
10	PSBeP-10	+	+	-		
11	PSBeP-	+	+	+	+	-

	11					
12	PFBeP-2	+	-	_	_	-
13	PFBeP-3	+	-	-	-	-
14	PFBeP-4	+	-	_	-	-
15	PFBeP-5	+	-	-	-	-
16	PFBeP-6	+	+	_	-	-
17	.PFBeP-7	+	+	-	-	-
18	PRBeP-1	+	+	-	_	-
19	PRBe+P-2	+	+	_	-	-
20	PRBeP-3	+	+	-	-	-
22	.PRBeP-5	+	+	+	-	-
23	PRBeP-6	+	+	+	+	-
24	PRBeP-7	+	+	+	_	-
25	PRBeP-8	+	+	-	_	-
26	PRBeP-9	+	+	-	-	-
27	PRBeP-10	+	+	+	+	-

4.3 Salt stress tolerance of selected alkalotolerant bacterial endophytes:

Several halophillic or halo tolerant microorganisms are known to adapt and grow under wide range of salt (NaCl) concentrations. Generally, the NaCl tolerance exhibited by the microorganisms is far higher than that of other organisms; hence microorganisms are potential candidates in mitigating salt stress in agricultural soils. Therefore, in order to explore the salt tolerance limits of the selected eleven alkalo tolerant bacterial endophytes, during present study an in vitro plate assay was carried out using varying concentrations of NaCl(0-12%, w/v). The endophytes differed in their abilities to grow under salt stress conditions and none of the isolate could grow at or above 10% NaCl concentration, although only one isolate PRBeP-6 showed a very little growth at this concentration. All

the test isolates showed growth upto NaCl concentration of 4%, but only four isolates(PRBeP-6.PRBeP-10,PSBeP-8) could grow upto 6% NaCl. While only three isolates(PRBeP-6.PRBeP-10,PSBeP-8) were found tolerant to 8% concentration. In this way only four alkaliphic isolates (PSBeP-8,PSBeP-11,PRBeP-6, PRBeP-10) also exhibited salt tolerance ability displaying the halo-alkalo properties. Adaptation of bacteria to high salt concentration is mainly due to maintenance of intracellular balance of sodium ions, secretion of osmolytes, changing cell physiology according to external environmental conditions.(Sleather and Hill,2001).

Table 4.3.*In vitro* selection of alkalo-tolerant bacterial endophytes for salt stress tolerance based on growth abilities at different salt concentrations during plate assay:

Sl.no	Bacterial Isolates	*In vitro* growth at different salt concentration(%,w/$_v$)							
		0	2.0	4.0	6.0	8.0	10.0	12.0	14.0
1	PSB_eP-1	+	+	+	+	-	-	-	-
2	PSB_eP-2	+	+	+	+	-	-	-	-
3	PSB_eP-3	+	+	+	+	-	-	-	-
4	PSB_eP-4	+	+	+	+	-	-	-	-
5	PSBeP-8	+	+	+	+	+	+	-	-
6	PSBeP-9	+	+	+	-	-	-	-	-
7	PSBeP-11	+	+	+	+	+	±	-	-
8	PRBeP-5	+	+	+	+	-	-	-	-
9	PRBeP-6	+	+	+	+	+	±	-	-
10	PRBeP-7	+	+	+	+	-	-	-	-
11	PRBeP-10	+	+	+	+	+	+	-	-

4.4 Plant growth promoting traits of halo-alkalo- tolerant bacterial endophytes.

All the selected eleven bacterial endophytes were further screened for their plant growth promoting traits (*In vitro*) such as Phosphate (P) solublization, IAA production, siderophore production, Ammonia production, HCN production, urease production and protease production.

4.4.1 Phosphate (P) - solublization

During in vitro plate assay for solublization using mineral salt medium amended with insoluble phosphorus source, tri calcium phosphate (TCP) as the only phosphorus source, all the selected eleven bacterial endophytes were found to solubilize insoluble phosphate sources. All the eleven isolates but at varying extents,(Plate 4.1) among the isolates the four bacterial endophytes (PRBeP-6,PRBeP-10,PSBeP-8,PSBeP-11) showed better P- solubilizing activity as compared to other cultures (Table 4.4),. as was evident by formation of larger clear zone around the colonies. Solublization of insoluble form of phosphorus is a critical aspect of increasing soil phosphorous availability(Rodriguez and fraga,1999).The application of phosphate solubilizing bacteria(PSB) as bioinoculants simultaneously enhance phosphorous uptake by the plant and crop yield(Mehta and Nautiyal,2001). Bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *enterobacter* and some others are well known to solubilize the insoluble phosphate compounds and help in plant growth (Frey-klett *et al*., 2005). It is reported that endophytic bacteria solubilize the inorganic phosphorus by reducing pH through the excretion of organic acid, while organic phosphorous is solubilized by various extra cellularly produced microbial phosphatases and phytases thereby, resulting in improved plant growth and development (Rodriguez and Fraga, 1993). Several Similar PGPB isolates belonging to *pseudomonas* and *bacillus* were also found solubilize insoluble phosphate in soil. (Saravankumar *et al*., 2011).

4.4.2 Phytohormone (IAA production):

.Among the eleven bacterial isolates tested for *in vitro* IAA production, all the isolates except for three (PSBeP-4,PSBeP-8 and PRBeP-7) showed the positive result by developing the cherry red color in tryptophan containing culture tubes (Table 4.4).Comparatively higher IAA production was shown by isolates PRBeP-6 and PRBeP-10 as was evident by the intensity of the red color. IAA functions as a main plant growth regulatory hormone (phytohormones), controlling many important physiological processes including tissue differentiation, root initiation and elongation, cell enlargement and division and responses to light (Leveau and Lindow, 2005). Therefore, IAA production by any endophytic microorganism is crucial for plant growth. IAA-producing microorganisms increase the overall development and enlongation of plant roots, which constitutes a greater root surface area that enabling the plant to get more nutrients from the soil.(Boiero , *et al*., 2007)

4.4.3 Synthesis of metal chelating structures: Siderophore production test:

Among eleven bacterial isolates (PSBeP-1,PSBeP-2,,PSBeP-8,PSBeP-9,PSBeP-11, PRBeP-6,PRBeP-10) were confirmed to produce siderophore by CAS-blue agar assay.(Table 4.4).The three isolates(PSBeP-8,PRBeP-6 and PRBeP-10) showing larger orange zone formation on CAS agar plates indicated higher siderophore producing ability. These five isolates were able to produce the color change from blue to orange. Siderophores and other metal chelating compounds directly promote the growth of the plants by providing iron and other nutrients and thus stimulating the biosynthesis of the other antimicrobial compounds by the bacteria that suppress the growth of pathogenic organisms such as *Fusarium oxysporum and Rizactonia solani*. These compounds thus function as stress factors in inducing host resistance (Haas and Defago, 2005). Siderophores specifically bind the iron molecule and provide them to the plant at the same time depleting this micronutrient in the environment thereby restricting the growth of the pathogens (Dorjey *et al*., 2017). CAS agar plate assay has been reported as an ideal method for identification of siderophore (iron chelating compounds) by several bacterial isolates Seong and Shin, (1996).

4.4.4 Volatile compound (HCN) production:

HCN production has been shown to play both beneficial and harmful effects on plants. (Cattelan *et al*., 1999).Generally, plant growth promoting bacteria produce synthesis certain volatile compounds, such as HCN as a means of their biocontrol activity. Therefore the selected test bacterial endophytes evaluated in vitro for HCN production by plate assay. Most of the cultures were found negative for HCN production while, only four bacterial endophytes (PSBeP-1, PRBeP-5, PRBeP-7) showed positive HCN production.(Plate 4.1.a) The production of HCN in excess may play a critical role in the control of fungal diseases in seedlings (Flaishman *et al*., 1996). Blumer and Haas (2000) have also reported the role of HCN in plant defense regulation against phytopathogens. Plant growth promoting bacteria have attracted much attention as biological control agents for their role in combating plant diseases, though their full potential has not yet been reached. Therefore, many of the bacterial endophytes promote plant growth indirectly by inhibiting the growth and activities of phytopathogens by the production of antimicrobial substances like HCN through a variety of different mechanisms.(Bashan Y, de Bashan L, 2005).

4.4.5 Urease production

A positive urease activity indicated the ability of the bacteria to release ammonia in the surroundings that is utilized as nitrogen source by plants and microbes. Therefore the selected bacterial endophytes were examined for their in vitro urea hydrolyzing capabilities by plate assay.(Table 4.4). Only four bacterial endophytes (PSBeP-2,PSBeP-3,PRBeP-10, PSBeP-11) were found to be urease positive while rest of the other isolates could not hydrolyze urea indicating their incapability to synthesize urease enzyme.

4.4.6 Ammonia production

Ammonia can be produced by several processes such as ammonification, degradation and decarboxylation and deamination and urease-mediated hydrolytic degradation of urea.This ammonia released in soil through various bacterial activities produced by bacteria is taken up plants as a source of nitrogen for their growth. Considering the importance of ammonia production in soil through bacterial activity all the selected endophytes were evaluated in vitro for ammonia production. Only four bacterial endophytes PSBeP-1, PSBeP-4 PRBeP-5 and PRBeP-7 showed positive test for ammonia production. (Table 4.4) Ammonia production is as important as nitrogen fixation, because ammonia released upon hydrolysis of organic biomolecules serves as available nitrogen source in soil that is utilized by plants and microbes.

4.4.7 Protease production

Proteases are important hydrolytic enzymes secreted by several microorganisms which are involved in the breakdown of proteins present in soil organic matter and increasing its availability for the biological system the selected bacterial endophytes were evaluated for protease production and all were found positive for protein hydrolysis. (Table 4.4)

Based on various abiotic (pH/salt) stress tolerance and *in vitro* plant growth promoting traits, only superior bacterial endophytes (PSBeP-8.PSBeP-11, PRBeP-10, PRBeP-6) were selected for further studies.

Table 4.4.Plant growth promoting traits of bacterial endophytes based on qualitative testing by plate assay

Sl.no	Bacterial Isolates	*In vitro* plant growth promoting abilities						
		Phosphate-solublization	IAA production	HCN production	Urease production	Siderophore production	Ammonia production	Protease Production
1	PSB_eP-1	+	+	+	-	+	+	+
2	PSB_eP-2	+	+	-	+	+	-	+
3	PSB_eP-3	+	+	-	+	-	-	+
4	PSB_eP-4	+	-	+	-	-	+	+
5	PSBeP-8	++	-	-	-	++	-	+
6	PSBeP-9	+	+	-	-	-	-	+
7	PSBeP-11	++	+	-	+	+	-	+
8	PRBeP-5	+	+	+	-	-	+	+
9	PRBeP-6	++	++	-	-	++	-	+
10	PRBeP-7	+	-	+	-	-	+	+
11	PRBeP-10	+++	++	-	+	++	-	+

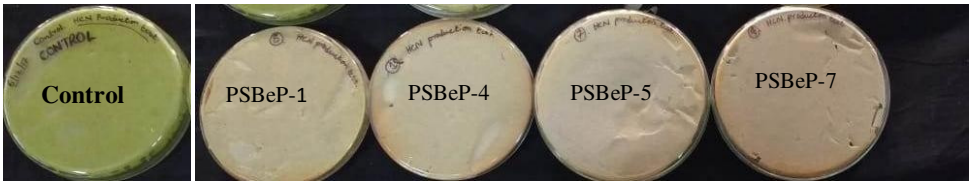


Plate 4.1(a). Endophytic bacterial isolates showing *In vitro* HCN production during qualitative testing by plate assay.

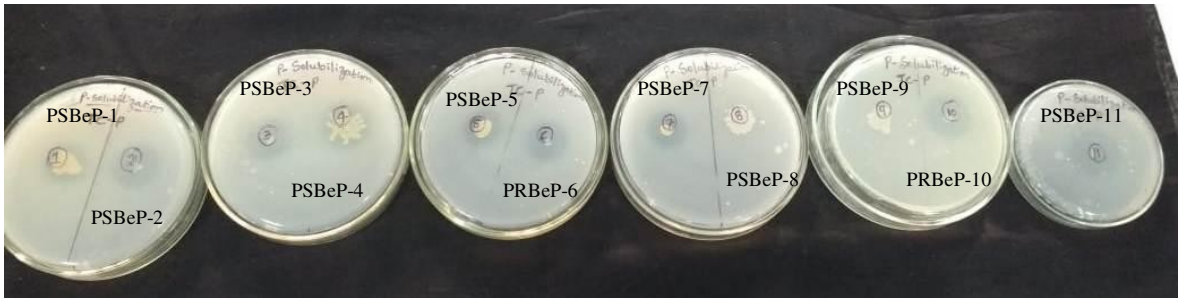


Plate 4.1(b): *In vitro* solublization of fixed phosphorous source (TCP) by endophytic bacterial isolates during qualitative testing by plate assay.

4.5 In vitro growth profile of bacterial endophytes under pH and salt stress conditions

Out of eleven native endophytic bacterial strains isolated from different regions of *Phyllanthus* plant during present study, four alkalo halo tolerant isolates(PSBeP-8.PSBeP-11,PRBeP-10,PRBeP-6) were selected on the basis of qualitative ability to grow under alkaline stress conditions (pH 7.0-10.0) and salt stress conditions (0-12%,w/v NaCl).and their *in vitro* plant growth promoting traits during plate assay. All the four alkalo halo tolerant strains were further evaluated for their growth potentialities under pH and salt stress conditions in liquid media (*In vitro*).The isolate PRBeP-6 showed maximum growth by attaining 1.5 O.D. by 24 h of incubation. It exhibited growth up to pH 10.0. The isolate PRBeP-8 showed maximum growth by attaining 1.8 O.D at pH 7(Fig.4.1.b).The growth decreased with increasing pH; although, it showed growth up to pH 10.0 with extended lag phase. Another bacterial isolate PRBeP-10 also exhibited maximum growth at pH 7.0 with (1.041 O.D) (Fig.4.1.c) at 48 h. The isolates also showed very little growth at pH 11.0; but the growth decreased with increasing alkaline levels. and bacterial isolate PSBeP-11 also showed the maximum growth at pH 7.00 with 1.21 O.D(4.1.d) it exhibited growth up to pH 10 with decreasing trend along with increasing pH gradient. Simultaneously, all these four selected bacterial isolates were also screened for salt tolerance using different (0-14%) concentration of NaCl. The isolate PRBeP-6 exhibited a good salt tolerance ability upto 6 % NaCl concentration and exhibited maximum growth at 0 % NaCl at 48 h incubation (Fig.4.2.a) period. A gradual decreasing trends were observed along the increasing salt concentration gradient in all cases. The isolate PRBeP-8 showed the salt tolerance up to 10% NaCl concentration but maximum growth at 0% and better growth upto 6 %, concentration comparatively the isolate showed better halo-tolerance compared to(Fig.4.2.b)PRBeP-10 isolate exhibited growth upto 10% of NaCl concentration, with maximum growth at 0% and also growth curve shows the decreasing trend along the increasing salt gradient.(Fig.4.2.c).PSBeP-11 shows minimum salt tolerance ability, it showed growth upto 6% NaCl concentration and started showing decreasing trend for further increase in salt concentration(Fig.4.2.d).Although, higher alkaline and saline tolerance for fluorescent *Pseudomonas* and *Bacillus* sp. has been reported well by earlier workers (McMillan *et al* 2010; Qu *et al*., 2011). But most of the studies have shown maximum alkalotolerance limit up to pH 10 for most of the *Pseudomonas* strains

(Bhakthavatchalu *et al* 2013). However, present investigation explored better osmo alkalotolerant endophytic bacterial isolates depicting growth up to pH 10.and salt concentration up to 12% (NaCl,w/v) the tolerance of bacteria in stress conditions were determined. These properties may be helping host plants living in stress conditions by interactions of bacteria and plants. In the present study, the maximum salt tolerance of endophytic bacteria was observed at 2.5% and 7.5% salt concentration. It is noteworthy here that that the growth rate decreased with the increased in salt concentration. All bacterial isolates were tolerant with 2.5% sodium chloride, while none of the isolate tolerated 15% salt concentration. *E. cloacae* showed a high salt tolerance rate with a growth at 10% salt concentration, while the other two showed good growth at 7.5%, which is similar to previous studies (Egamberdieva D, *et al*., 2008). The highly salt tolerant bacteria along with plant growth promoting traits would be advantageous for use in the mitigation of salt stress to make cultivation possible in saline agriculture lands (Egamberdieva D, *et al*., 2008).

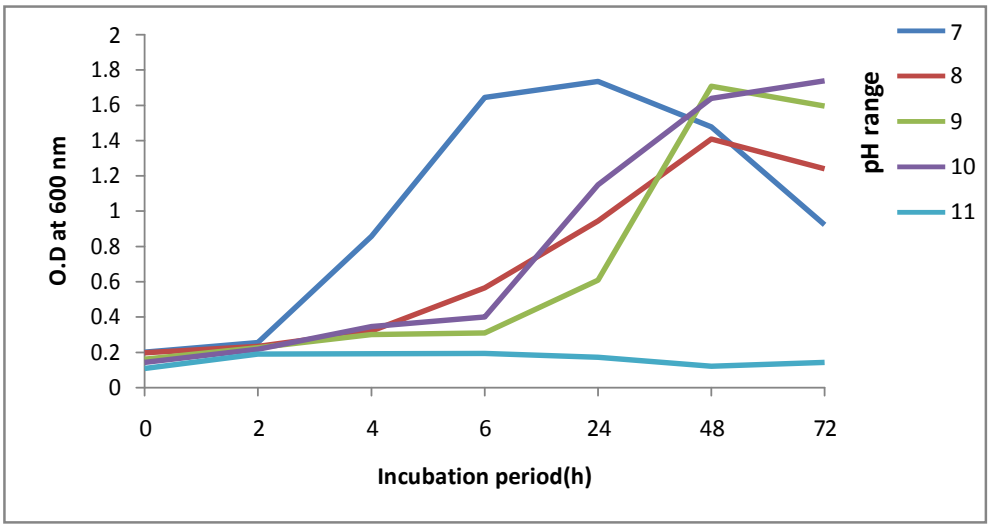


Fig.4.1, (a): Growth profile of the selected bacterial endophyte PRBeP-6 under alkaline pH conditions in broth cultures.

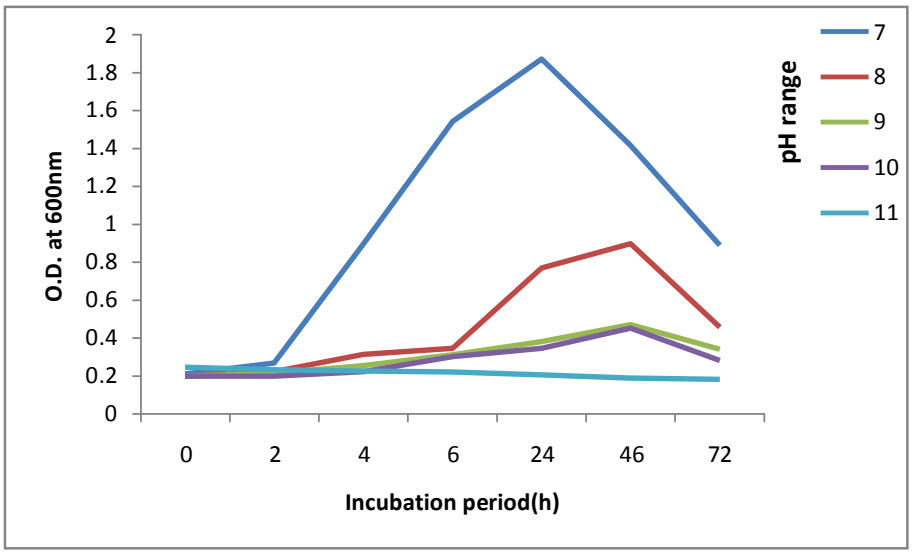


Fig.4.1, (b) Growth profile of the selected bacterial endophyte PSBeP-8 at different alkaline pH conditions in broth cultures.

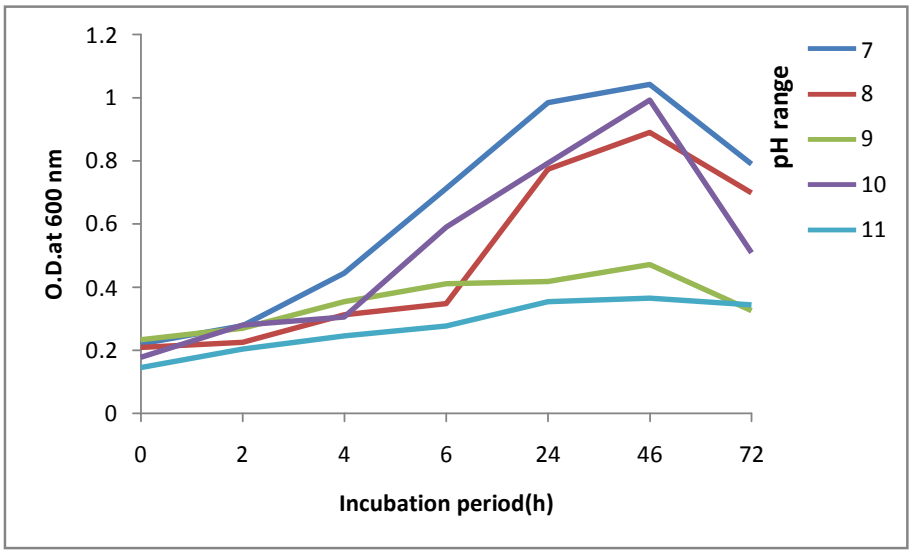


Fig.4.1, (c) Growth profile of the selected bacterial endophyte PRBeP-10 under alkaline pH conditions in broth cultures

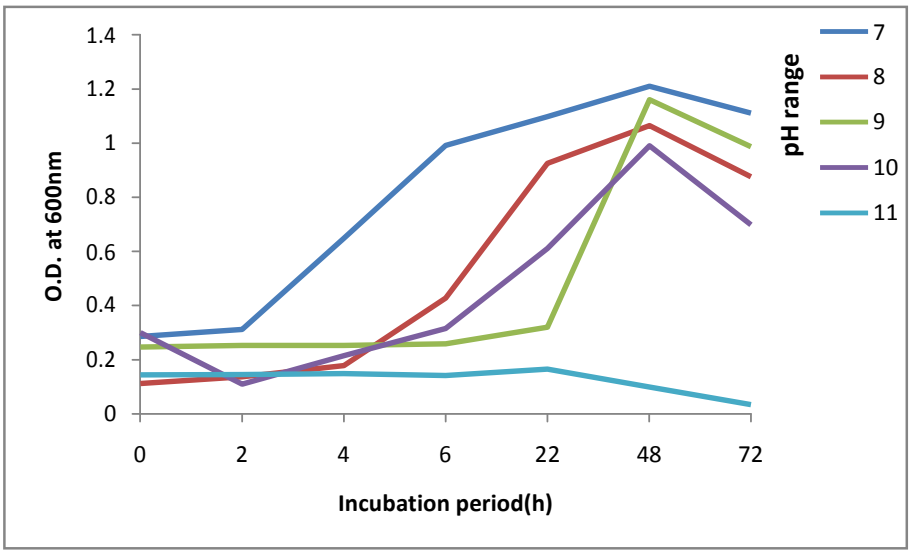


Fig.4.1, (d): Growth profile of the selected bacterial endophyte PSBeP-11 under alkaline pH conditions in broth cultures.

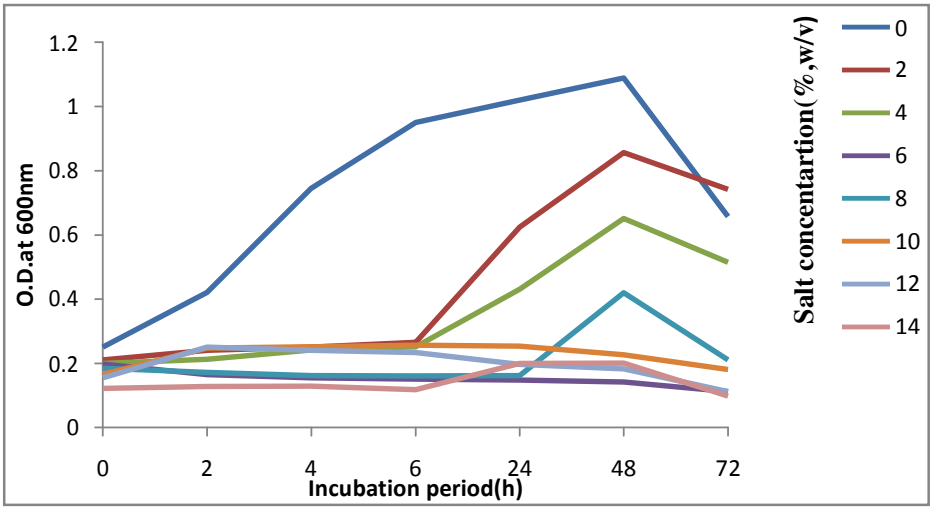


Fig.4.2. (a). Growth profile of the bacterial endophyte (PRBeP-6) at different salt (NaCl) concentrations in broth cultures

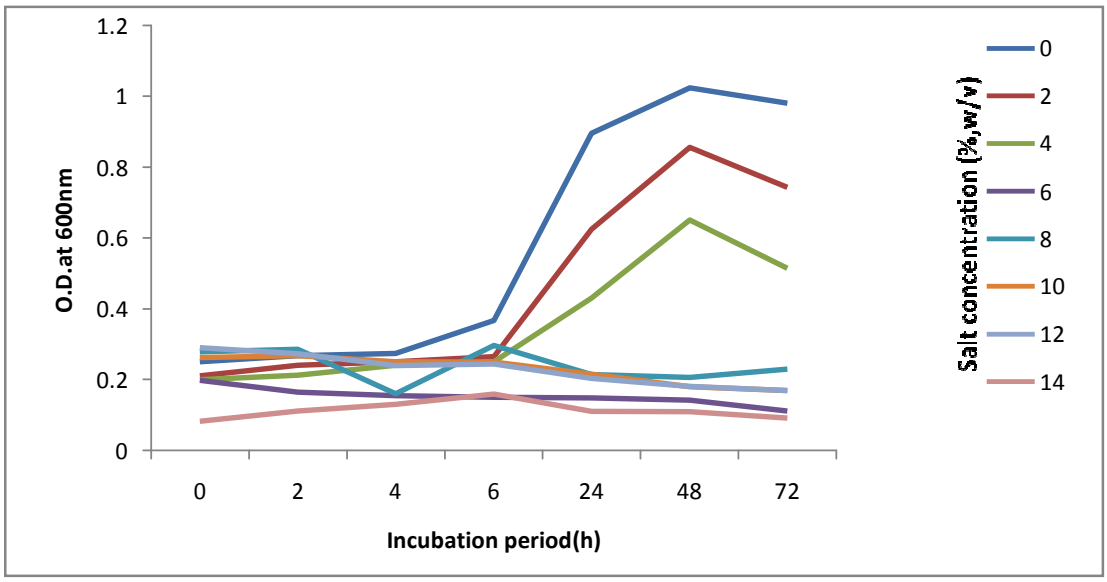


Fig.4.2.(b). Growth profile of bacterial endophyte (PSBeP-8) at different salt (NaCl) concentrations in broth cultures

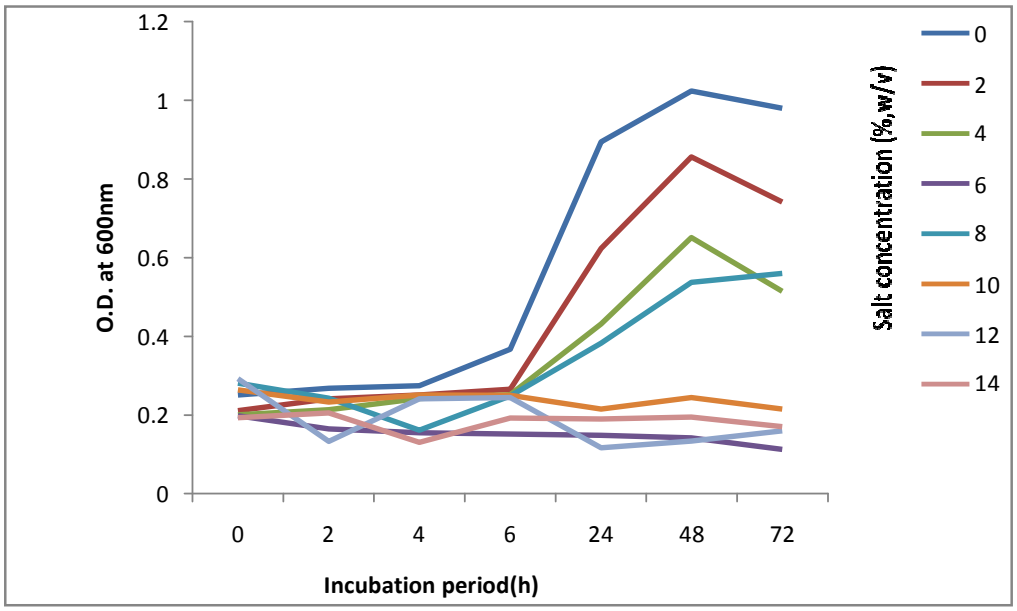


Fig.4.2.(c). Growth profile of bacterial endophyte (PRBeP-10) at different salt (Nacl) concentrations in broth cultures.

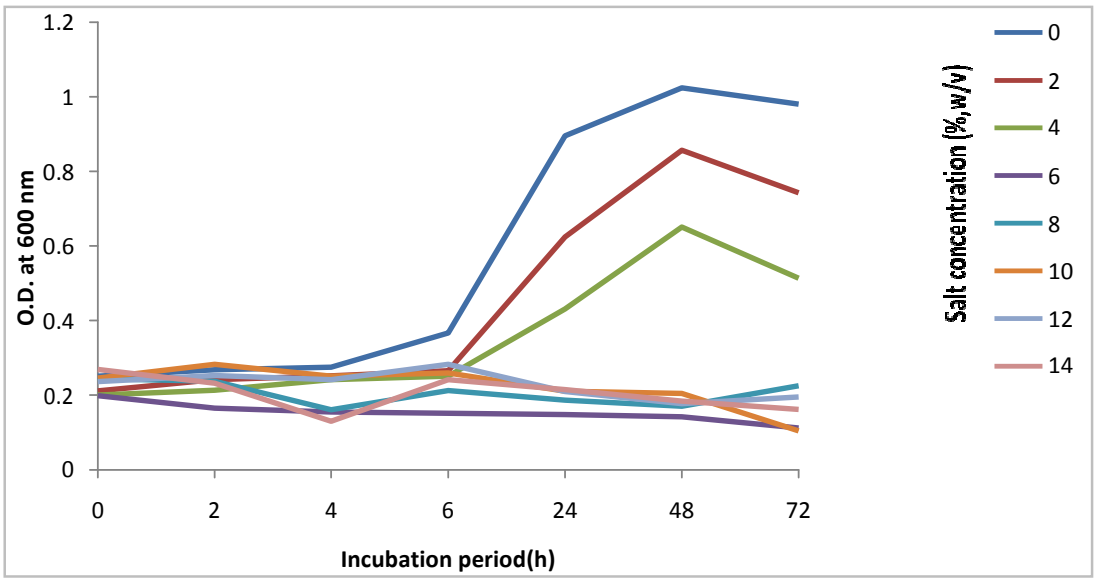


Fig.4.2. (d). Growth profile of the bacterial endophyte (PSBeP-11) at different salt (NaCl) concentrations in broth cultures

4.6 P-solublization under abiotic (alkaline) stress conditions

After preliminary screening of bacterial isolates for P- solublization, the selected cultures PRBeP-6, PRBeP-10, PSBeP-8, and PSBeP-11 were further evaluated for p-solublization quantitavely in P deficient mineral salt medium amended with tri calcium phosphate(TCP) as the only P-source. Under normal conditions (pH 7.0), significantly higher P- solublization was recorded for the isolate PRBeP-6 (269.27 µg/mL) that was followed by the isolates PSBeP-8 (223.52 µg/mL), PRBeP-10(220.61 µg/mL) and PSbeP-11(211.42 µg/mL) at 72 h,(Fig4.3 a,b,c,d) the values being non significant(Table 4.5).All the isolates released highest phosphate in culture filtrate at 72 h period under normal conditions, indicating 72 h as the optimum period for P- solublization. As the pH level increased, the phosphate released in culture filtrate also showed a gradual decrease. At pH 9.0, maximum phosphate released in culture filtrate was recorded for the isolate PSBeP-8 (95.62 µg/mL) followed by the isolate PRBeP-10 (81.18 µg/mL) at 72 h period that was significantly higher than those for other cultures (Fig 4.3 b and c). The P- solubilizing potential of bacterial endophytes was gradually decreased at higher alkaline conditions. At pH (10.0), maximum phosphate was recorded for the isolate PRBeP-10 (66.60 µg/mL) followed by the isolate PSBeP-8 (60.56 µg/mL) at 72 h. (Fig 4.3 c and b) .

The results revealed clearly that the isolates PSBeP-8 and PRBeP-10 having high p-solubilizing potential under normal conditions and also retaining it under alkaline pH stress conditions were found superior and thus were identified as potential PSB's. It is well known that Various P- solubilizing bacteria(PSB) play a vital role in P availability from both organic and mineral sources in soil (Iyer & Rajkumar 2017).This role is attributed to the ability of PSB to produce low-molecular-weight acids such as formic, acetic, propionic, lactic, glycolic, fumaric and succinic acid which use their carboxyl and hydroxyl groups to chelate cations such as Ca^{+2} and Mg^{+2}(Rashid, 2004);(Al-Enazy,2017).This chelation solubilizes insoluble soil phosphorus also. Phosphate solubilizing organisms are well known to well known to adopt various mechanisms for p- solublization such as synthesizing of metal chelating compounds ionophores, synthesis of enzymes (Phosphataes, phytases). Chelation, acidification and exchange reactions are primarily responsible for P release from insoluble sources by PSB (Gulati, *et al* ,2004) (Oliveira, *et al*.2009).

Table 4.6. Phosphate concentrations released upon solublization of TCP in the culture* filtrates of the selected bacterial isolates under alkaline stress conditions.

Bacterial isolates	Incubation period(h)	Concentration of solubilized phosphate(µg/mL) under different pH conditions				S.Em/CD # at 5%			
PRBeP-6		7.0	8.0	9.0	10.0		a	B	a*b
	24	144.41	76.70	51.01	49.46	S.Em ±	2.56	2.56	5.139
	48	189.12	80.63	50.18	35.21		7.04	7.04	14.804
	72	269.27	96.71	50.94	33.36	CD			
	96	100.30	56.36	43.36	30.39	(5%)			
PRBeP-10	24	128.84	60.22	49.90	34.67	S.Em ±	0.804	0.804	1.60
	48	201.26	92.36	64.01	61.18				
	72	220.61	95.01	81.18	66.60	CD			
	96	90.26	55.18	55.60	45.97	(5%)	2.31	2.31	4.63
PSBeP-8	24	128.72	91.91	42.80	34.53	S.Em ±	11.14	11.14	22.28
	48	141.31	128.12	69.01	59.60		32.09		
	72	223.52	136.81	95.62	60.56	CD			
	96	90.52	82.41	63.80	40.18	(5%)		32.09	64.18
PSBeP-11	24	129.31	90.16	50.83	44.35	S.Em ±	1.27	1.27	2.55
	48	204.09	130.43	55.53	43.22		3.67	3.67	9.89
	72	211.42	121.6	50.22	37.70	CD			
	96	98.96	91.6	56.77	39.60	(5%)			

*The cultures were grown in mineral salt medium .amended with TCP (10 g/L) at 28 ± 2 ^{0} c for 3 d under different pH conditions.

#a-pH; b-incubation period; a*b-interaction

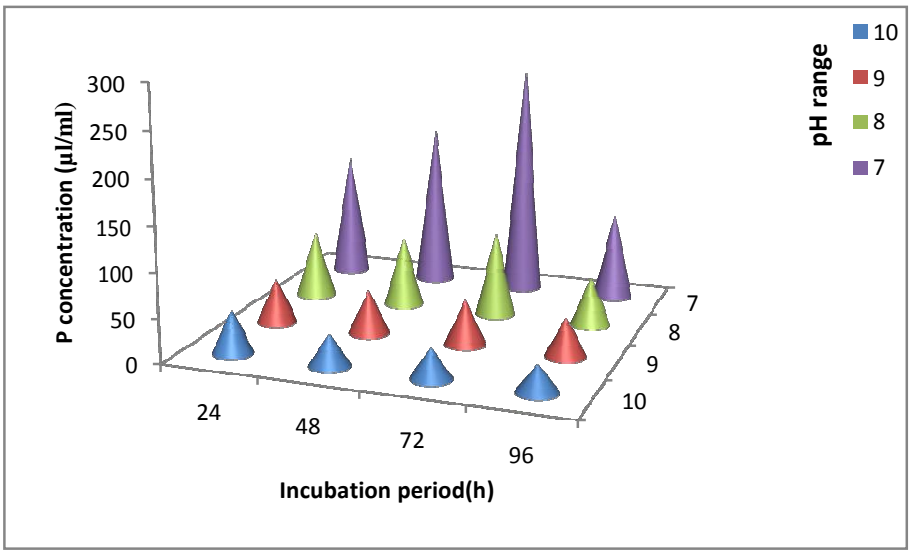


Fig 4.3 (a) Concentration of phosphate released upon solublization of tricalcium phosphate in the culture filtrate of the bacterial endophyte PRB$_e$P-6 under abiotic (alkaline pH) stress conditions.

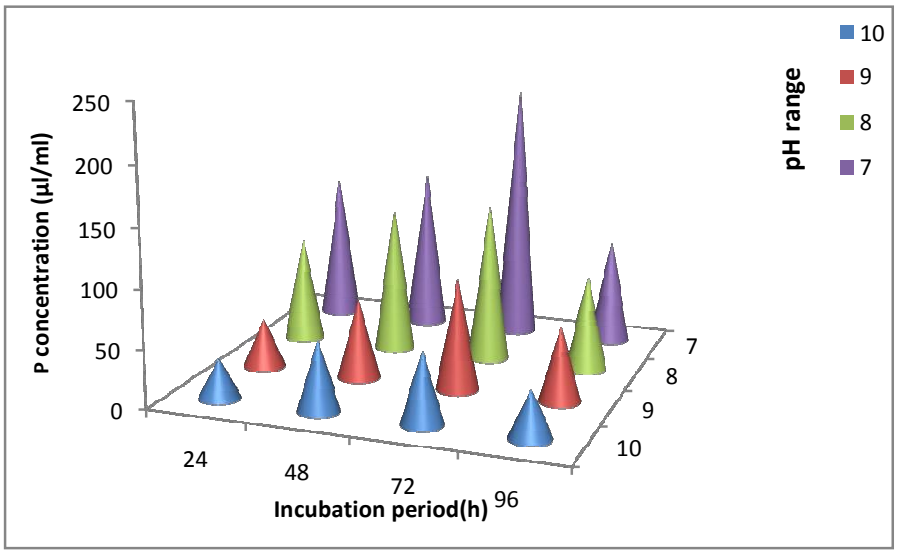


Fig 4.3 (b): Concentration of phosphate released upon solublization of tricalcium phosphate in the culture filtrate of the bacterial endophyte PSB$_e$P-8 under abiotic (alkaline pH) stress conditions.

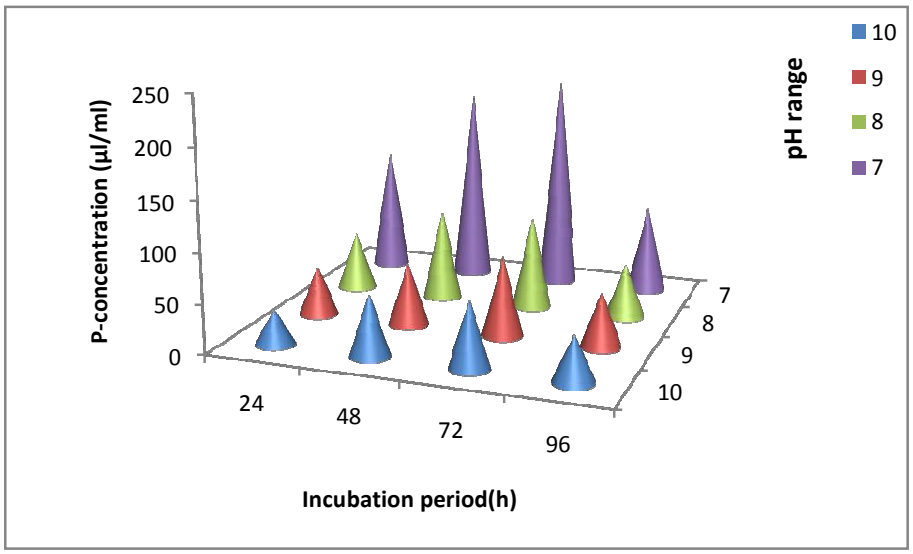


Fig 4.3(c): Concentration of phosphate released upon solublization of tricalcium phosphate in the culture filtrate of the bacterial endophyte PRB$_e$P-10 under abiotic(alkaline pH) stress conditions

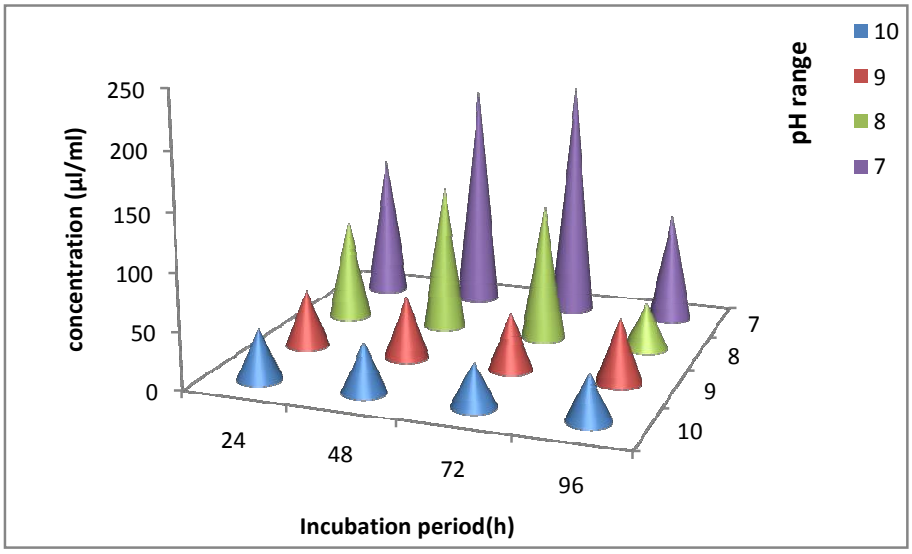


Fig 4.3(d): Concentration of phosphate released upon solublization of tricalcium phosphate in the culture filtrate of the bacterial endophyte PSB$_e$P-11 under abiotic(alkaline pH) stress conditions

4.7 P-solublization under Salt stress conditions

The selected endophytic bacterial cultures were evaluated for P-solublization activity at salt stress conditions (0-10% NaCl, w/v).Under normal conditions,(0%, NaCl), the maximum and significantly higher phosphate concentration was recorded for the isolate PSBeP-8 (87.16 µg/mL) followed by the isolate PRBeP-10 (83.22 µg/mL) that was significantly higher phosphate concentration for cultures at 72 h period. All the four selected isolates released maximum phosphate in their culture filtrates at 72 h period of incubation. With increasing salt concentration increased, the P-solublization potential of the bacterial endophyte also decreased considerably. No phosphate solublization by any culture could be seen at 12% NaCl concentration, At 8% NaCl concentration the isolate PSBeP-8 exhibited significantly higher phosphate concentration (60.63 µg/mL) followed by the isolate PRBeP-10(44.63 µg/mL) at 72 h. (Table 4.6).Under salt stress conditions, the phosphate solublization by bacterial cultures was shown up to the maximum NaCl concentration of 10% while at higher NaCl concentration(12%) no phosphate could be estimated in the culture filtrate up to 96 h. At 10 % NaCl concentration the isolate PRBeP-10 exhibited highest phosphate concentration (38.47 µg/mL) followed by the isolate PSBeP-8 (34.15 µg/mL) at 72 h period of incubation.(Fig 4.4 c and b).The isolates PRBeP-6 and PSBeP-11 showed least P- solublization at 10% NaCl concentration(Fig 4.4 a and d).

In vitro phosphate solubilizing abilities of the isolates under abiotic stress(pH/salt) conditions can br attributed as a very important criteria for the alkaline/saline stress soil, since phosphorous availability is one of the major constraint limiting plant growth.Several strains of fluorescent *Pseudomonas, bacillus and Trichoderma* and *Aspergillus* have been reported as potential phosphate solubilizers in soil (Chagas *et al*., 2015) Therefore, it is clear that the microbes isolated from salt affected soils are capable to tolus solublization at higher saline conditions due to inoculation with phosphate solubilization as observed during present study as also reported by other workers. Kumar *et al.* (2010) In the present study, even at high salt concentration, all the PSB isolates confirmed noticeable levels of P-solubilization. The trait of enhanced phosphate solubilization in the presence of salt/ high pH might be of considerable significance for the survival of phosphate-solubilizing bacteria in alkaline soils.The strains should serve as an excellent model to study the physiological, biochemical, and molecular mechanism(s) of phosphate solubilization under

stressed ecosystems. Since the conditions prevailing in soil are much more complex than those under in vitro conditions, therefore, further studies(*In situ)* on the environmental factors affecting phosphate solubilization in saline/alkaline soils should suggest the basis for obtaining inoculants that are able to give greater benefits for crops of economic or agricultural importance, in tropical and subtropical areas. (Karthik *et al*., 2017). But the presence of salt-tolerant phosphate-solubilizing PGPR in soil is known to improve phosphate uptake by plants under such stress conditions (Zaidi *et al*., 2004).

Table 4.7. Concentration of phosphate released upon solublization of tricalcium phosphate (TCP), in the culture filtrates of selected bacterial isolates under salt stress conditions.

Salt concentration (%,w/v)	Bacterial isolates	Concentration of solubilized phosphate(µg/mL) under salt stress conditions at different time interval(h)				S.Em/CD # at 5%			
		24	48	72	96		A	B	a*b
0	PRBeP-6	49.7	53.36	64.63	50.36	S.Em ±	0.525	0.397	1.05
	PRBeP-10	44.77	50.55	83.22	71.32	CD #	1.487	1.124	2.975
	PSBeP-8	40.82	58.80	87.16	78.09				
	PSBeP-11	43.36	49.84	75.19	65.14				
2	PRBeP-6	46.88	51.95	63.22	72.77	S.Em ±	0.425	0.215	0.09
	PRBeP-10	36.88	53.36	62.22	50.55				
	PSBeP-8	19.98	69.56	72.09	70.55				
	PSBeP-11	54.07	68.14	63.22	58.55	CD # at 5%	1.281	1.051	1.346
4	PRBeP-6	39.00	41.25	51.67	30.95	S.Em ±	0.321	0.241	0.077
	PRBeP-10	42.59	48.77	53.22	49.14				
	PSBeP-8	37.59	54.77	63.22	49.64				
	PSBeP-11	45.47	53.36	57.45	47.02	CD # at 5%	1.124	1.024	1.150
6	PRBeP-6	29.56	30.26	31.45	20.30	S.Em ±	0.651	0.541	0.352
	PRBeP-10	44.56	34.07	45.19	31.55				
	PSBeP-8	32.52	48.43	62.52	37.30				
	PSBeP-11	22.06	34.77	41.81	25.14	CD # at 5%	1.24	1.021	1.26
8	PRBeP-6	26.88	28.22	33.08	10.49	S.Em ±	0.66	0.501	1.32
	PRBeP-10	27.02	32.98	44.63	11.60				
	PSBeP-8	26.32	42.66	60.63	20.60				
	PSBeP-11	23.36	30.55	32.37	21.66	CD # at 5%	1.88	1.42	3.76
10	PRBeP-6	15.90	11.11	15.63	1.25	S.Em ±	0.55	0.416	1.102
	PRBeP-10	24.91	30.36	38.47	12.25				
	PSBeP-8	15.47	28.36	34.15	10.25				
	PSBeP-11	11.95	14.77	28.15	5.28	CD # at 5%	1.56	1.18	3.12

*The cultures were grown in mineral salt medium .amended with TCP (10 g/L) at 28 ± 2 ^{0} c for 3 d under different salt conditions.

#a-salt concentration; b-incubation period; a*b-interaction

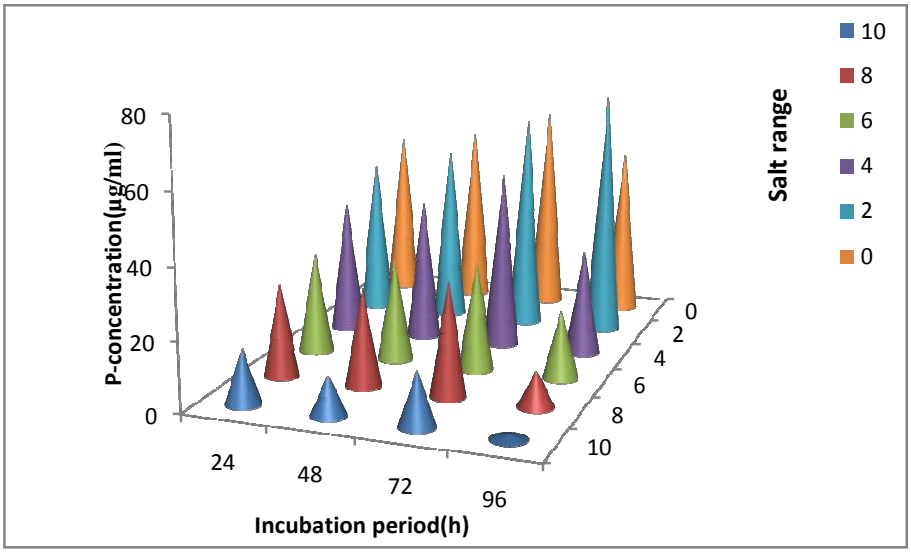


Fig.4.4 (a): Concentration of phosphate released upon solublization of tricalcium phosphate in culture filtrate of the endophytic bacterial isolate PRBeP-6 under abiotic (salst) stress conditions.

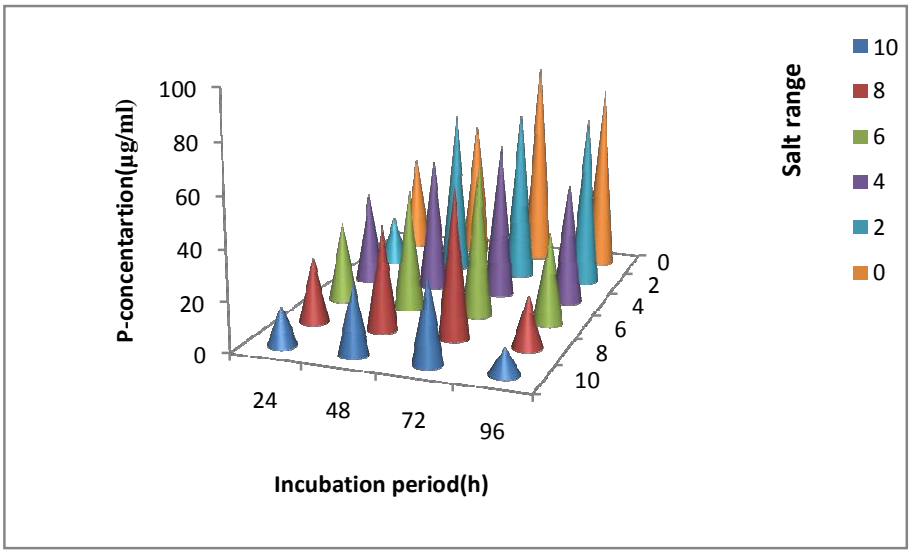


Fig.4.4 (b): Concentration of phosphate released upon solublization of tricalciu phosphate in culture filtrate of the endophytic bacterial isolate PSBeP-8 under abiotic (salt) stress conditions.

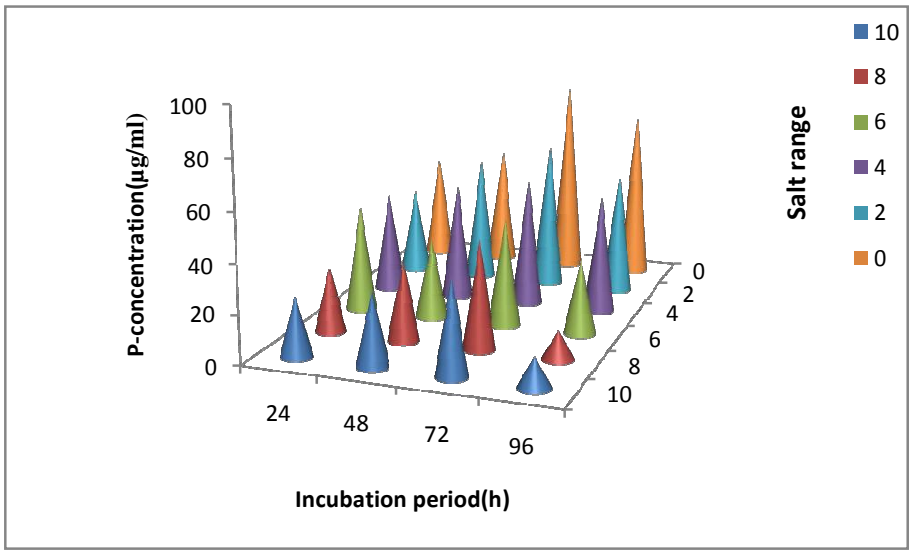


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Fig.4.4(c): Concentration of phosphate released upon solublization of tricalcium phosphate in culture filtrate of the endophytic bacterial isolate PRBeP-10 under abiotic(salt) stress conditions

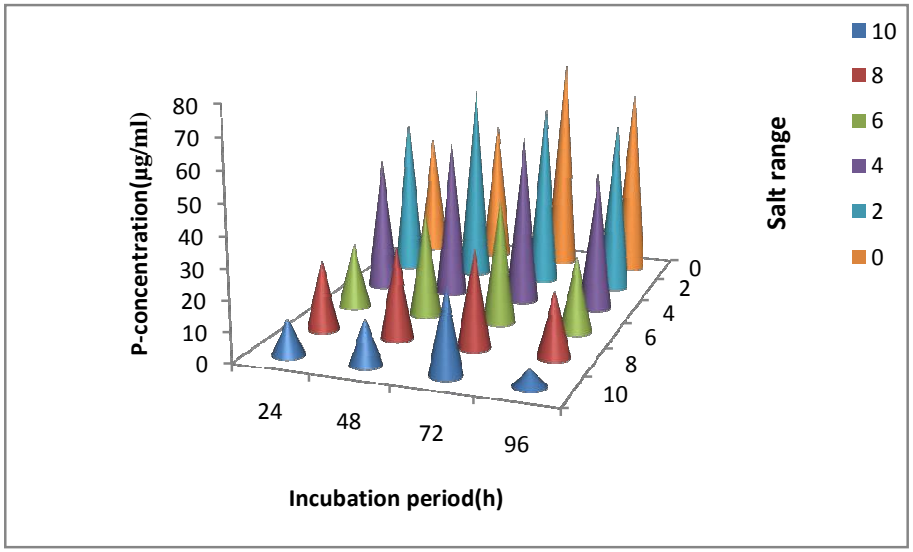


Fig.4.4 (d): Concentration of phosphate released upon solublization of tricalcium phosphate in culture filtrate of the endophytic bacterial isolate PSBeP-11 under abiotic (salt) stress conditions.

4.8 Siderophores production under alkaline stress conditions

The four selected bacterial endophytes which were detected for siderophore production on CAS agar during plate assay, were further evaluated for quantification of

siderophore in iron deficient liquid mineral salt medium Under normal pH(7.0) conditions, all the four isolates(PRBeP-6,PSBeP-8,PRBeP-10,PSBeP-11) exhibited higher siderophore production as compared to that under pH stress conditions.(Table 4.7). At neutral pH, the % sid units showed by various isolates in liquid broth cultures varied from 89.50 to 91.71 at 72 h showing their high siderophore producing abilities under normal conditions.(Table 4.7). But with increasing alkalinity the % sid units decreased gradually and minimum siderophore production was recorded at pH 10.0 in all the cases. All the isolates invariably showed maximum siderophore production at 72 h. At pH 8.0 maximum sid units recorded for PRBeP- 6 and PSBeP-11 respectively (85.33% and 85.33%)(Fig 4.5 a and d).At pH 9.0 maximum siderophore units were recorded in the culture filtrates of the isolates PRBeP-10(66.58%) at 72 h and PSBeP-8(59.81%) at 24 h.(Fig 4.5 c and b).Further decline in siderophore production at pH 10.0 was recorded at 72 h and only 46.78 and 48.78 sid units were recorded for PRBeP-6 and PRBeP-10 (Fig 4.5 a and c). However no siderophore units were recorded for higher pH values in any case. At extreme alkaline conditions i.e. at 9.0 and 10.0, all the cultures exhibited reduced siderophore production. The Isolates PRBeP-6 and PSBeP-11 although exhibited better siderophore production initially under normal pH conditions but they could not show high siderophore production under pH stress conditions, all the isolates during present study retained siderophore producing capabilities up to pH 10.0.

It is clearly evident from data that the bacterial endophytes were able to synthesis siderophores and solubilize phosphate under highly alkaline conditions. This finding suggests clearly their possible application as plant growth promoting bioinoculants for enhancing crop productivity in alkaline soils, potential application of PGP bacteria for agriculture has also been reported earlier by several workers. As pH plays an important role in the solubility of iron and their availability to the other organisms, hence the alkalotolerant isolates play a significant role in enhancing iron bioavailability in salt affected soils. (Saravankumar *et al*., 2011).

Table, 4.8: Siderophore production (*In vitro*) by the selected endophytic bacterial isolates under alkaline pH stress conditions*

Bacterial isolates	Incubation period(h)	siderophore units (%) produced by endophytic bacteria under different pH conditions at different time intervals(h)				S.E.m/CD # (5%)			
PRBeP-6		7.0	8.0	9.0	10.0		a	B	a*b
	24	78.65	76.57	46.76	42.40	S.Em	1.65	1.08	2.86

	48	90.48	83.04	46.41	43.15	± CD (5%)	4.71	3.08	14.50
	72	91.53	85.33	50.70	46.78				
	96	89.61	79.77	43.41	41.09				
PRBeP-10	24	78.95	58.60	23.93	13.59	S.Em ± CD (5%)	1.21 3.21	1.06 2.08	1.28 6.67
	48	88.05	70.62	48.80	17.59				
	72	90.55	82.23	66.58	46.58				
	96	85.69	74.20	43.40	23.27				
PSBeP-8	24	72.27	63.07	59.81	15.23	S.Em ± CD (5%)	1.08 2.14	0.654 1.21	0.706 2.58
	48	86.13	70.91	44.37	34.07				
	72	89.50	77.63	45.72	42.40				
	96	86.91	75.68	42.15	39.04				
PSBeP-11	24	83.20	72.01	14.02	9.18	S.Em ± CD (5%)	1.051 2.08	1.01 1.45	1.06 3.016
	48	90.05	78.02	21.23	33.31				
	72	91.71	85.33	41.38	35.73				
	96	89.77	79.86	39.86	34.07				

pH- (a), incubation period –(b): interaction(a*b)

* The cultures were in grown in iron deficient mineral salt medium at 28 ± 2^0c under alkaline pH stress conditions up to 96 h

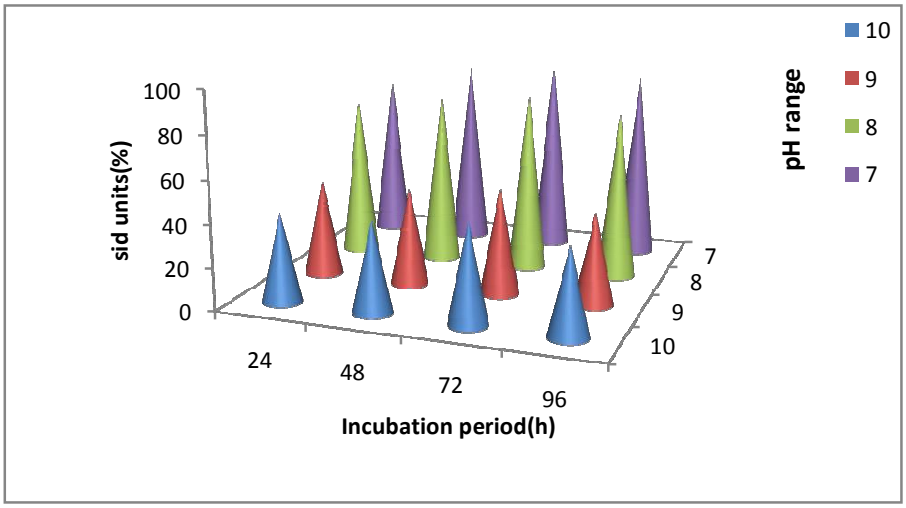


Fig.4.5 (a). Siderophore production (*In vitro*) by the endophytic bacterial isolate PRBeP-6 under abiotic (alkaline pH) stress conditions.

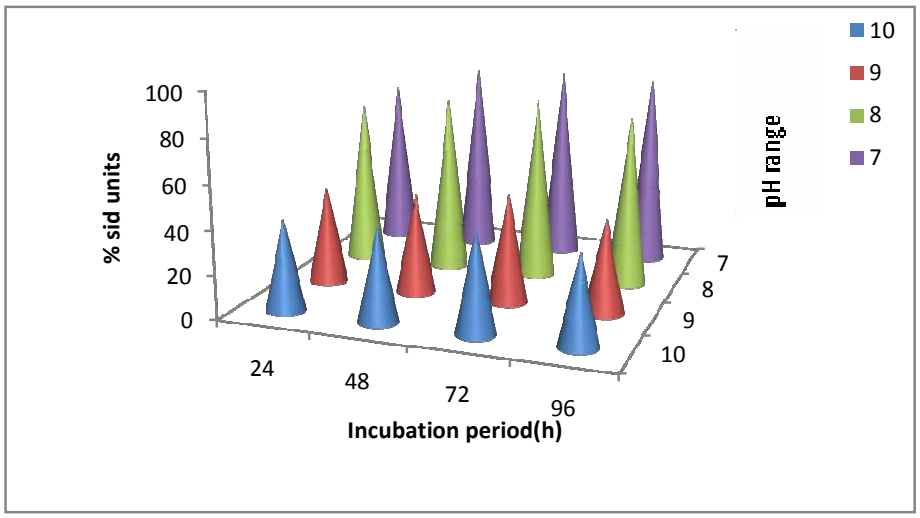


Fig.4.5 (b).Siderophore production (*In vitro*) by the endophytic bacterial isolate PSBeP-8 under abiotic (alkaline pH) stress conditions.

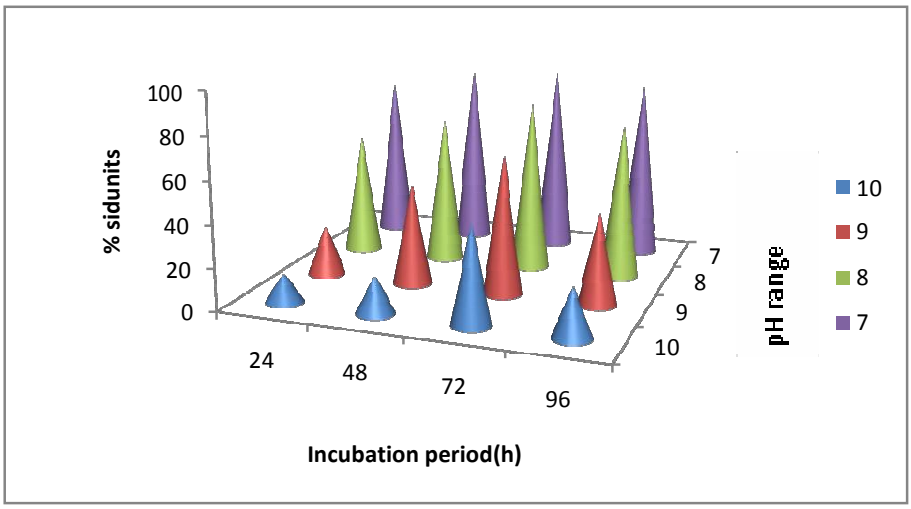


Fig.4.5(c). Siderophore production (*In vitro*) by the endophytic bacterial isolate PRBeP-10 under abiotic (alkaline pH) stress conditions.

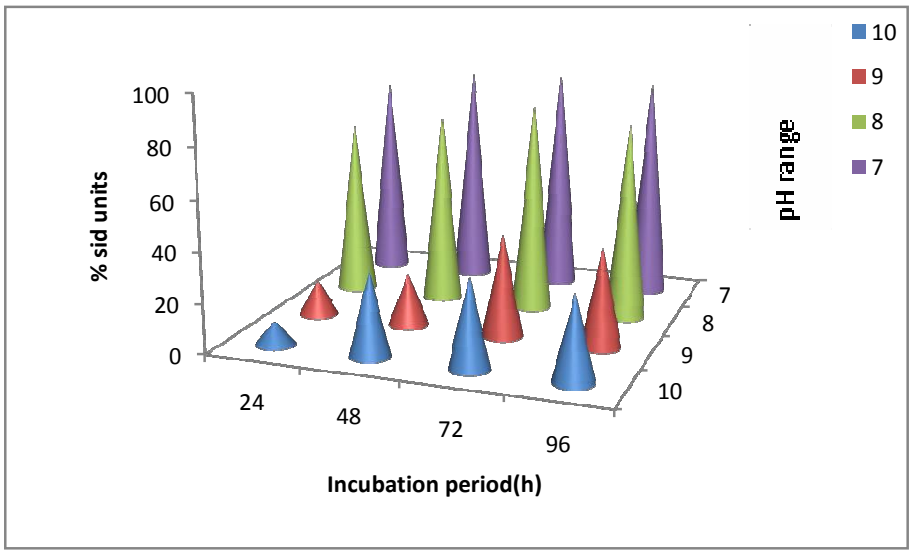


Fig.4.5(d).Siderophore production(*In vitro*) by the endophytic bacterial isolate PSBeP-11 under abiotic(alkaline pH) stress conditions.

Siderophore production under salt stress conditions:

The selected bacterial endophytes were also evaluated for siderophore production potential at saline conditions using mineral salt broth amended with varying concentrations (0-10%) of NaCl. All the four isolates exhibited good siderophore production activity under normal (0%, NaCl) concentrations and the percent sid units varied from 33.29 %(PRBeP-6) at 24 h to 77.15 % (PRBeP-10 and PSBeP-8) at 72 h (Table 4.8, fig 4.6.a). All the isolates produced highest sid units at 72 h period under normal conditions, indicating 72 h as the optimum period for siderophore production. As the salt concentration increased, the siderophore units in culture filtrates also decreased. At 8% NaCl concentration, maximum sid units(%)were recorded in case of the isolate PRBeP-10 (50.20%) followed by the isolate PSBeP-8 (38.20%) at 72 h(Fig 4.6 c and b).At 10% NaCl concentration maximum siderophore units(%) were recorded in the culture filtrates of the isolates PSBeP-8(28.33) followed the isolate PRBeP-10 (21.77).(Fig 4.6 b and c).Among all the strains tested, the isolate PRBeP-11 showed minimum % sid units at 10% NaCl concentration.(Fig 4.6.d). The data revealed clearly the high potential of the isolate PSBedP-8 and PRBeP-10 under normal and saline stress conditions.

The bacterial endophytes PRBeP-10 and PSBeP-8 exhibited much better siderophore synthesing abilities even under increasing NaCl concentration and retaining the ability up to 10% NaCl concentration. After conducting, proper in situ studies both the bacterial isolates from *Phyllanthus* sp might be expected to have a greater potential in ameliorating the salt stress in agricultural soils if used as bioinoculants. Dileep Kumar and Dube (1992) reported that inoculation of chick-pea and soybean seeds with a siderophore producing *Pseudomonas* sp increased germination, growth and yield of the plants. They have also reported an increased siderophore production under saline conditions. Recently, a relationship between reduction of Fe concentration and salt stress in soil has been very reported. This could be due to the negative effect of salt on enzymes implicated in iron uptake (M'sehli *et al*. 2010).

Table 4.9: **Siderophore production (*In vitro*) by the selected endophytic bacterial isolates under salt stress conditions**

Bacterial isolates	Incubation period(h)	siderophore units (%) produced by endophytic bacteria under different pH conditions at different time intervals(h)							S.E.m/CD # (5%)		
		0	2.00	4.00	6.00	8.00	10.00		a	b	a*b
PRBeP-6	24	33.29	29.37	21.26	18.07	11.00	5.67	S.E m ±	1.021	1.21	1.23
	48	45.61	40.57	30.83	24.00	17.17	11.36				
	72	53.97	48.69	39.65	36.66	29.18	15.98				
	96	40.12	35.45	26.82	24.41	16.94	10.87	CD # at 5%	0.89	1.05	0.93
PRBeP-10	24	54.41	48.07	39.08	35.49	32.11	21.77	S.E m ±	1.041	1.02	1.06
	48	66.55	59.27	55.98	54.54	48.91	15.15				
	72	77.15	43.35	69.37	65.37	50.20	18.30				
	96	36.77	33.15	32.88	31.42	28.38	12.30	CD # at 5%	0.75	0.62	0.465
PSBeP-8	24	44.08	39.20	30.95	28.41	16.66	15.77	S.E m ±	1.08	0.956	1.032
	48	46.31	42.83	40.00	36.63	29.33	14.80				
	72	77.15	53.35	48.27	45.37	38.20	28.83				

	96	34.77	38.15	32.86	22.42	18.38	12.30	CD # at 5%	0.56	0.89	0.498
PSBeP-11	24	38.78	33.83	24.47	32.01	23.70	1.20	S.E m ±	1.021	0.89	0.90
	48	35.50	47.54	41.56	37.20	21.41	4.00				
	72	51.75	54.93	46.72	43.27	28.14	12.18				
	96	36.82	34.74	23.11	18.29	13.55	1.00	CD # at 5%ss	0.98	0.745	0.730

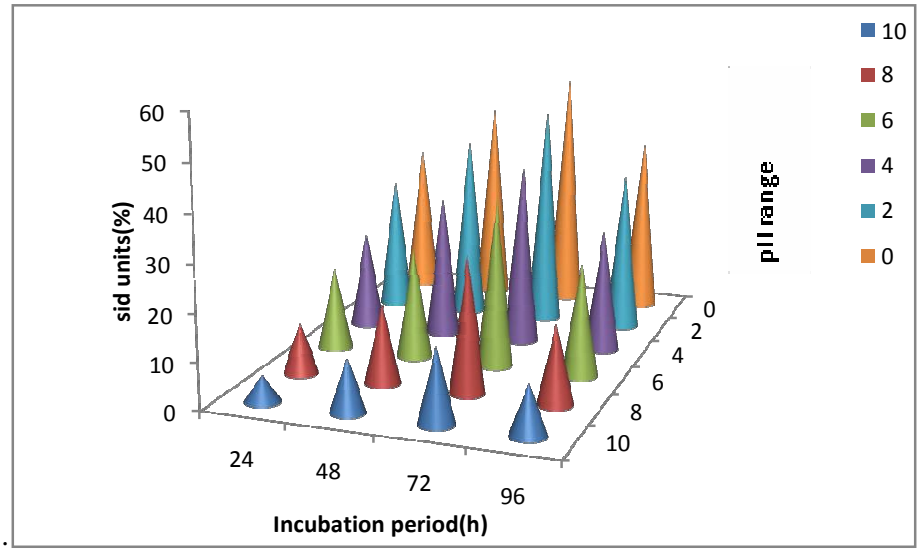


Fig.4.6(a).Siderophore production(*In vitro*) by the endophytic bacterial isolate PRBeP-6 under abiotic(salt) stress conditions.

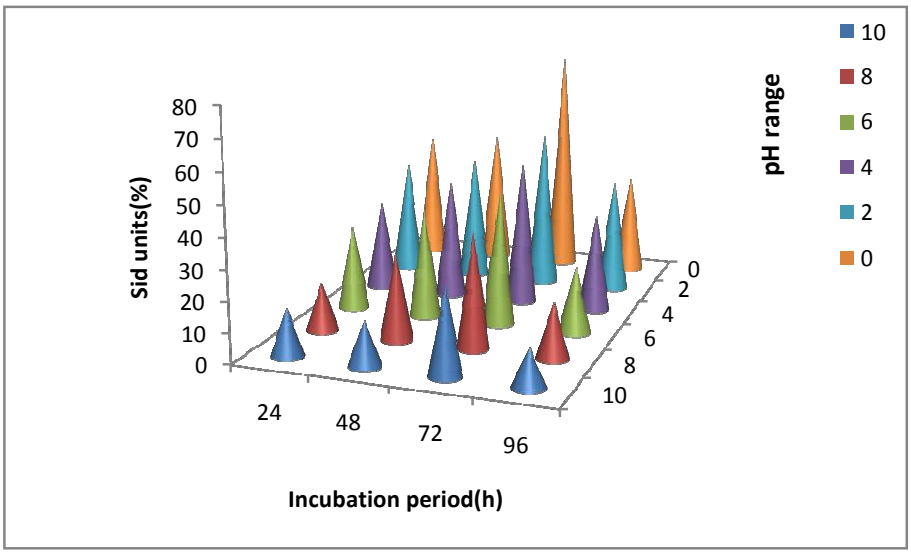


Fig.4.6 (b).Siderophore production (*In vitro*) by the endophytic bacterial isolate PRBeP-8 under abiotic(salt) stress conditions.

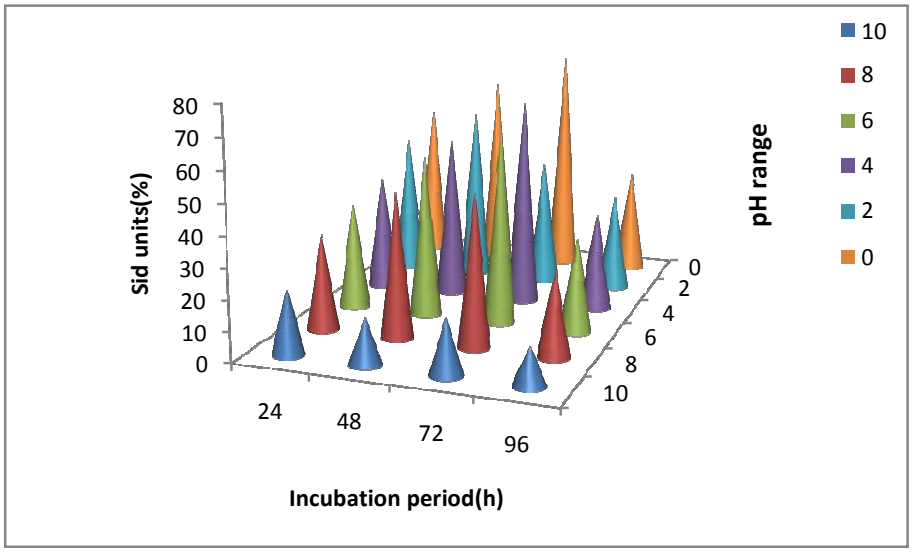


Fig.4.6(c). Siderophore production (*In vitro*) by the endophytic bacterial isolate PRBeP-10 under abiotic (salt) stress conditions

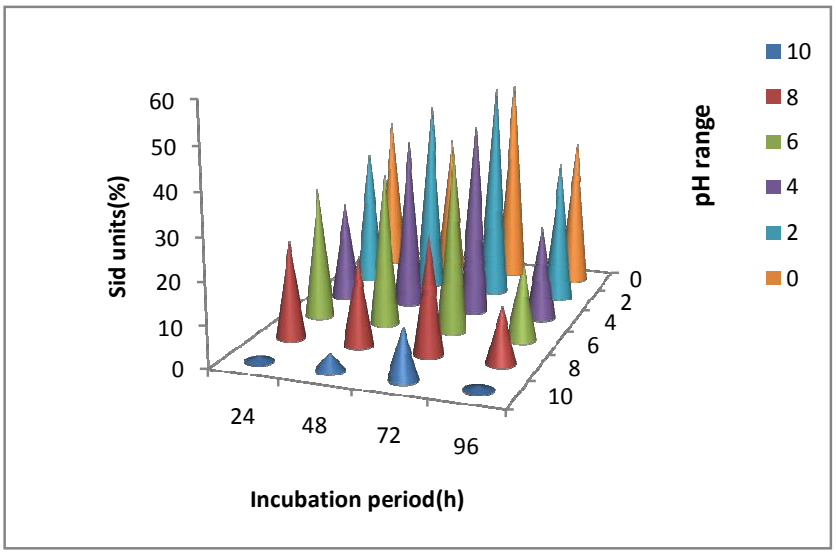


Fig.4.6(d).Siderophore production(*In vitro*) by the endophytic bacterial isolate PSBeP-11 under abiotic(salt) stress conditions

4.10 Influence of bacterial inoculation on fenugreek (Trigonella *foenum graceum*) seed germination:

In vitro viability test for germination of fenugreek seeds (var. Pusa early bunching) under normal and abiotic stress conditions was carried out by plate assay using wet paper towel. Influence of inoculation of two native bacterial isolates (PSBeP-8 and PRBeP-10) on seed germination under normal pH and salt(7.00 and 0mmol NaCl,) concentrations, under pH stress conditions (pH 8.00-10.00) and at salt stress (50 mmol-300 mmol NaCl) conditions was studied up to 3d.

4.10.1 Seed germination under normal conditions:

The germination percentage of fenugreek seeds was determined by growing the sterilized seeds on glass petri plates fitted with paper towel soaked in distill water as test solution (pH 7.00, NaCl 0%). Clear differences were observed in the seed germination rates among inoculated and un inoculated treatments.(Table 4.9).Fenugreek seeds normally germinate after 3-4 d of sowing under normal soil conditions and it can also resist moderate levels of alkalinity and salinity in the soil(Zhu, 2007). Under normal pH (7.0)conditions the germination of uninoculated seeds, was only 66.67% which was raised 98.33 and 86.67 % for the seeds inoculated with PSBeP-8 and PRBeP-10 respectively after 3 d.(Fig 4.7). The

bacterial inoculation significantly enhanced seed germination at different time intervals. An increase of 47.5% and 29.9% in seed germination due to inoculation with the isolates PSBeP-8 and PRBeP-10 respectively after three days of germination was recorded.

4.10.2 Seed germination under alkaline pH stress conditions:

In vitro viability test for seed germination with or without bacterial inoculation was carried out under alkaline pH stress conditions ranging from pH 8.0-10.0 up to 3 d. Under stress conditions the bacterial inoculation increased germ inability of the seeds. At pH 8.0, germination of the seeds inoculated with the bacterial isolates PSBeP-8 and PRBeP-10 was 78.34 and 68.34 % respectively as compared to 51.67 % germination in case of uninoculated control after 3 d (Table 4.9).

without any inoculation, the fenugreek seeds showed germination only up to the alkaline pH of 8.00 while no seed germination were recorded at higher pH values (9.00 - 10.00)(Table 4.9 and Fig 4.7) However a significant increase of % seed germination due to bacterial inoculation under alkaline pH stress(pH 9.0 and 10.0) conditions was recorded over corresponding controls under the same stress conditions. At pH 9.00, seed germination was increased to 68.34%(PSBeP-8) and 58.34 % (PRBeP-10) compared to 0 % germination in uninoculated control after three day. At the alkaline pH of (pH 10.0), bacterial inoculation increased the seed germination was increased from 0% (uninoculated control) to 35 % (PSBeP-8) and 38.34% (PRBeP-10) after three d.(Table 4.8).Usually fenugreek seeds can't tolerance high levels of alkaline pH conditions during germination period.(Egamverdieva, 2013.) But, inoculation of bacterial endophytes confers the required mechanisms for alkaline stress tolerance, enhancing the germination rates. Depicting variable beneficial effects of the bacterial endophytes on seed germination of fenugreek seeds, the bacterial isolate PRBeP-10 showed better effect on seed germination. Fenugreek plants can tolerate moderate levels of alkalinity under field conditions, but higher levels of alkalinity are known to be detrimental to its growth and development (Egamberdieva, 2013).Present study stands out to be a novel approach in determining the effects of bacterial endophytes on germination of fenugreek under alkaline stress conditions.

Table 4.9. Influence of bacterial endophyte inoculation on germination of fenugreek (*Trigonella foenum-graceum*) seeds under alkaline stress conditions.

S.No	Bacterial isolates	pH condition	seed germination(%) at different time intervals (h)			S.E.m/CD # at 5%			
			24	48	72		a	b	a*b
1	PSBeP-8	7	55	95	98.33	SEm±	1.49	1.29	2.59
		8	46.67	76.67	78.34				
		9	23.34	55	68.34	CD(5%)	4.36	3.78	7.56
		10	11.67	18.34	35				
2	PRBeP-10	7	85	96.67	86.67	SEm±	1.24	1.075	2.51
		8	54.34	61.67	68.34				
		9	38.34	71.66	58.34				
		10	18.34	21.67	38.34	CD(5%)	3.62	3.14	6.28
3	Un inoculated (control)	7	40	50	66.67	SEm±	2.693	2.33	4.66
		8	23.34	31.67	51.67				
		9	0	0	0	CD(5%)	7.86	6.80	13.11
		10	0	0	0				

pH-(a), Time interval-(b), a*b- interaction.

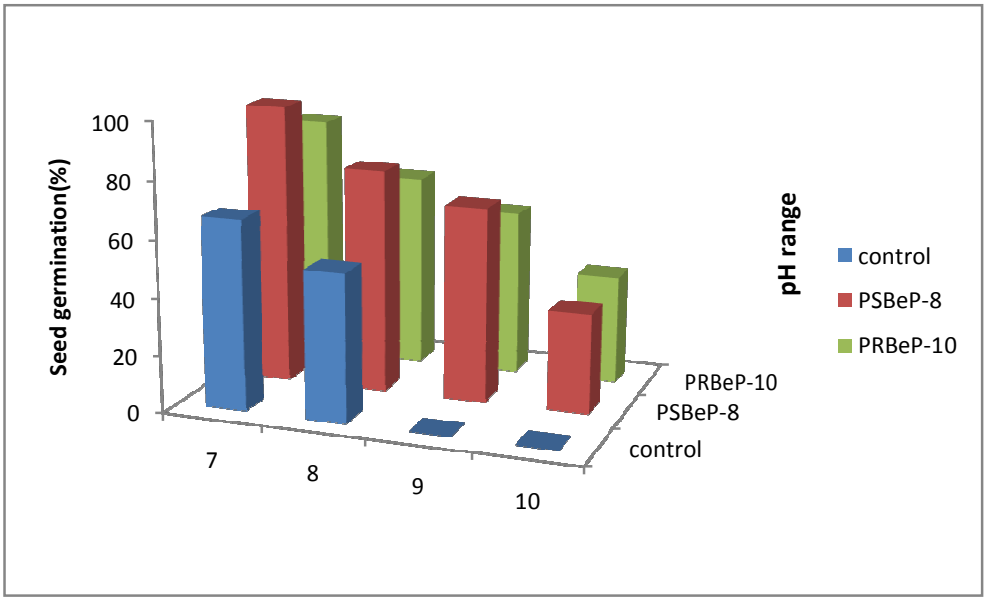


Fig.4.7.Influence of endophytic bacterial inoculation on germination of fenugreek (*Trigonella foenum-graceum*) seeds under alkaline stress conditions.

4.10.3 Under salt stress conditions

In order to test germ inability of fenugreek seeds under normal and saline conditions with or without bacterial inoculation, plate assay was performed using paper towel soaked with distill water for distill water for normal conditions, NaCl solutions for saline stress conditions in vitro the seed germination under saline conditions, seed germination by plate assay).Under normal conditions at (0 mMol NaCl) concentration the germination of uninoculated seeds was 66.67% which was increased to 96.67% and 98.34% by inoculation with the isolates PSBeP-8 and PRBeP-10 respectively at 3 d(Table 4.10) .With increasing concentration of NaCl from 0- 300 mmol a gradual decrease in seed germination but it was severely affected in case of uninoculated seeds. Without any inoculation, the fenugreek seeds showed germination only up to 100 mMol NaCl concentration while no seed germination was recorded at high saline conditions at (>150 mmol) NaCl concentration, (Table 4.10 and Fig 4.8). At NaCl concentration of 250 mmol NaCl concentration, seed germination was increased to 42.34% (PSBeP-8) and 52.34% (PRBeP-10) compared to 0% germination in uninoculated control after three d. (Table 4.10 and Fig 4.8).At highly saline condition (NaCl 300 mmol) seed germination was increased to 38.34 % (PSBeP-8) and 58.34 % (PRBeP-10) compared to 0% germination in case of uninoculated control after three days(Table 4.10).

Thus, results of the study indicate clearly the enhancement of fenugreek seed germination under saline stress conditions induced by the of bacterial inoculation on seed germination is clearly established the beneficial effect of the selected cultures on the seed germination of fenugreek seeds. Similar results were reported by earlier workers in which seed germination was decreased slightly with increasing salt concentrations (from 75 to 125 mM NaCl), compared to the control seeds (water only). The observed seed germination was 89 ± 4.6% with distilled water and 76 ± 3.5% at 75 mM NaCl, 61 ± 4.9% at 100 mM NaCl and 47 ± 3.9% at 125 mM NaCl concentration (Asari *et al*., 2017).Salt stress adversely affects the seed germination. However the inoculation of bacterial cultures positively affected the seed germination of fenugreek seeds under salt stress conditions. Hence the selected endophytic bacterial isolates had positive effect on the seed germination of fenugreek seeds under saline conditions when compared to the seed germination of uninoculated seeds. In another study it was observed that halo-tolerant bacteria have direct

and indirect effect on paddy seed germination under salinity stress conditions (Jha and Subramanian, 2013). This might be due to the inoculated bacteria which supplement and stimulate IAA production in plant which has a crucial role in seed germination. Under saline stress conditions, desiccation tolerant strain *Micrococcus luteus* showed an improved growth of maize by HCN and IAA production which restore and protect maize growth (Raza and Faisal, 2013). PGPR through the augmentation of IAA under salinity stress cause reduction in the uptake of toxic ions, and resulting in the improved plant growth (Zhang *et al*., 2008, Chakra borty *et al*., 2011). There is also a significantly positive correlation between bacterial salt and osmotic tolerance. Surprisingly, the same bacterial strains capable of surviving at high osmotic stress can also tolerate high salinity conditions indicating dual adaptation as osmotic and salinity stress induce the same initial limiting conditions (Wood *et al*., 2015)

Table.4.10. Influence of bacterial inoculation on germination of fenugreek (*Trigonella foenum-graceum*) seeds under salt stress conditions.

S.No	Bacterial endophytes	Salt concentration(mmol)	seed germination(%) at different time periods(h)			S.Em/CD # at 5%			
			24	48	72		A	B	a*b
1	Isolate PSBeP-8	0	38.34	41.67	96.67	Sem±	1.45	0.952	2.51
		50	48.34	58.34	68.34				
		100	38.34	46.34	62.34				
		150	28.34	48.34	58.52				
		200	25	44.34	46.67				
		250	25	38.34	42.34				

		300	18.34	28.34	38.34	CD(5%)	4.152	2.71	7.191
2	Isolate PRBeP-10	0	48.34	88.34	98.34	Sem±	1.408	0.922	2.439
		50	40	48.34	75.00				
		100	38.34	68.34	72.34				
		150	31.67	65.12	68.00				
		200	21.67	62.12	62.34				
		250	20.00	58.34	52.34				
		300	19.67	56.67	50.34	CD (5%)	4.02	2.63	6.96
3	Uninoculated control	0	28.34	66.67	66.67	Sem±	1.65	1.082	2.86
		50	13.34	43.34	66.67				
		100	0	16.67	11.67				
		150	0	0	0				
		200	0	0	0				
		250	0	0	0				
		300	0	0	0	CD (5%)	4.71	3.08	8.71

salt concentration-a, time period-b, interaction-a*b.

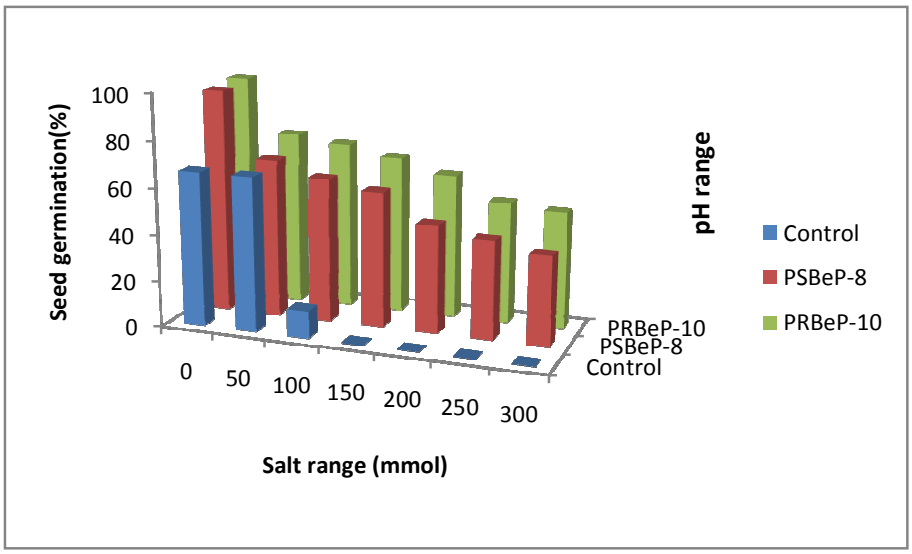


Fig.4.8. Influence of endophytic bacterial inoculation on germination of fenugreek (*Trigonella foenum-graceum*) seeds under salt stress conditions..

4.11. Effect of bacterial inoculation on performance of leafy vegetable fenugreek under salt stress conditions: *In vivo* study

Based on in vitro pH and salt tolerance, various plant growth promoting abilities**,** p-solublization, and, siderophore production, under salt and pH stress conditions, ability to enhance seed germination, two superior bacterial endophytes (PSBeP-8 and PRBeP-10) were selected for studying influence of seed bacterization on vegetative growth and green biomass yield and growth of fenugreek under normal and saline stress conditions. Pot culture experiments were performed using saline soil with pH 9.0, electric conductivity of 5.95, available phosphorous 19.60 kg/ha and organic carbon 0.42%; to compare the biomass yield under saline stress conditions the experiment was also carried out using normal soil with pH 7.24, electrical conductivity 2.00, available phosphorus 30.10 kg/ha organic carbon 0.49% (Table 4.11).The growth of the leafy vegetable was studied in pots using three sets of soil: normal soil, half saline soil(Saline soil : sand :: 1:1) and saline soil with four levels of bacterial inoculation(uninoculated control and inoculated with the isolate PSBeP-8, PRBeP-10 and their co-cultures(PSBeP-8+PRBeP-10).All the experiment were conducted in triplicates and observations were recorded for shoot length and fresh shoot weight after 15 and 30 days of germination.15 seeds were sown in each pot and only 6 plants were maintained after germination in each case.

Under normal soil conditions, significant difference in the growth attributes of inoculated fenugreek plants as compared to uninoculated plants were recorded.(Plate 4.2) Maximum increase in shoot length and fresh shoot weight of fenugreek was reported in case of inoculated with co-cultures(PsBep-8+PrBep-10) (3.56 cm in normal soil, 3.16 cm in half saline soil and 3.26 in saline soil a 15 d of germination. Fresh weight of shoots also increased due to bacterial inoculation in all the cases and maximum values were recorded for the co-cultures in normal soil (0.099 g, half saline soil 0.066 g and 0.093 g in saline soil at 15 d of germination. After 30 d of germination, further increase in the shoot length was recorded, attaining 6.13 cm (normal soil), 5.36 cm (half saline soil and 5.26 cm (saline soil) height in plants inoculated with the co-cultures as compared to co-inoculated as compared to the height in their respective uninoculated controls(Table 4.12).Similarly maximum fresh shoot weight was recorded for the plants inoculated with co-cultures showing 0.50 gm in normal soil, 0.60 gm in half saline soil and 0.59 gm in saline soil as compared to their respective controls under corresponding soil conditions(Table 4.12,Fig 4.9)

It is clearly evident from the results that the co-inoculation of PSBeP-8 and PRBeP-10 performed better as compared to their mono-cultures by enhancing plant height and leafy biomass yield. At both the stages of germination (at 15 and 30d), the co-inoculated plants showed increase in growth parameters as compared to the uninoculated control as well as their plants inoculated with monocultures. Under saline conditions the uninoculated plants experienced more adverse effects of salt stress on them and exhibited more retardation in growth, yellowing of leaves and reduction in seed germination, seedling growth, shoot length and overall growth of plants, as compared to their inoculated plants. These effects were clearly visible among inoculated and un-inoculated plants also.(Plate 4.2).The selected cultures PSBeP-8 and PRBeP-10 were found to be effective in mitigating the harmful effects caused by the salt stress on the fenugreek plants, hence the shoot length and fresh shoot weight of fenugreek plants were increased after the inoculation with the monocultures and co cultures of the bacterial isolates indicating the potential application of these isolates in agriculture as bioinoculant in future. The increased performance of the bacterial isolates may also be attributed to might also attributed due to their excellent *in vitro* plant growth promoting traits which were studied extensively in this study (Fig.4.9) The increase in the plant growth parameters can be co-related due to prolific production of phytohormones, such as IAA, gibberellins, cytokinins. And increased bio-availability of

the major and minor nutrients (Kim *et al*., 2014). Previous studies on plants performed under salt stress in general with 100 mM NaCl) have also shown that plants inoculated with bacterial strains have higher growth rate than plants not inoculated with bacteria. In a study performed on peanuts (*Arachis hypogaea*), inoculation with *Brachybacterium saurashtrense*, *Brevibacterium casei* and *Haererohalobacter* increased the growth of plants in comparison to the control plants. (Kim *et al*., 2014).Similar results have been reported for radish inoculated with *Staphylococcus kloosii* and *Kocuria erythromyxa*, lettuce inoculated with *Bacillus subtilis*, *B. atrophaeus*, *B. spharicus*, *S. kloosii* and *K. erythromyxa*, strawberry inoculated with *B. subtilis*, *B. atrophaeus*, *B. spharicus*, *S. kloosii* and *K. Erythromyxa* and wheat inoculated with *P. rifietoensis* under salt stress.

Table 4.11. Characteristic features of the soil used for pot culture experiments..

Soil chemical properties	Normal soil	Saline soil
pH	7.24	9.0
Electrical conductivity(EC)	2.00dsm^{-1}	5.95dsm^{-1}
Available nitrogen	211.91 kg/ha	188.02 kg/ha
Available phosphorous	30.10 kg/ha	19.60 kg/ha
Avaliable potassium	283.30 kg/ha	283.30kg/ha
Sodium ions	8.71 me/l	37.83 me/l
Potassium ions	1.78 me/l	1.61me/l
Calcium ions	4.20 me/l	8.50 me/l
Magnesium ions	5.90 me/l	9.25 me/l
Chloride ions	6.00 me/l	26.50 me/l
Sulphate ions	4.29 me/l	8.56 me/l
Sodium adsorption ratio	3.88 mmol^{-1}	12.70 mmol^{-1}
Organic carbon(O.C)	0.49 %	0.42 %

Table.4.12. Various growth attributes of leafy vegetable fenugreek after 15 and 30 days of germination under abiotic stress conditions using normal,half-saline an saline soil during pot culture experiment.

Soil condition	Treatments	Growth parameters at two stages of germination			
		Shoot length(cm)	Fresh shoot weight(gm)	Shoot length(cm)	Fresh shoot weight(gm)
I.Normal soil	i.Uninoculated control	2.66	0.013	3.26	0.12
	ii.Isolate PSBeP-8	3.06	0.11	5.03	0.42
	iii Isolate.PRBeP-10	3.13	0.023	5.16	0.31
	iv.Co-inoculation PSBeP-8+ PRBeP-10	3.56	0.099	6.13	0.50
II.Half saline soil (Saline soil:sand) (1 : 1)	i.Uninoculated control	1.93	0.036	3.80	0.17
	ii.Isolate PSBeP-8	3.70	0.063	5.16	0.43
	iii Isolate.PRBeP-10	2.30	0.096	5.20	0.38
	iv.Co-inoculation PSBeP-8+ PRBeP-10	3.16	0.066	5.36	0.60
III.Saline soil	i.Uninoculated control	1.86	0.046	3.13	0.18
	ii.Isolate PSBeP-8	2.60	0.083	4.23	0.46
	iii Isolate.PRBeP-10	3.26	0.086	4.16	0.56
	iv.Co-inoculation PSBeP-8+ PRBeP-10	3.20	0.093	5.26	0.59

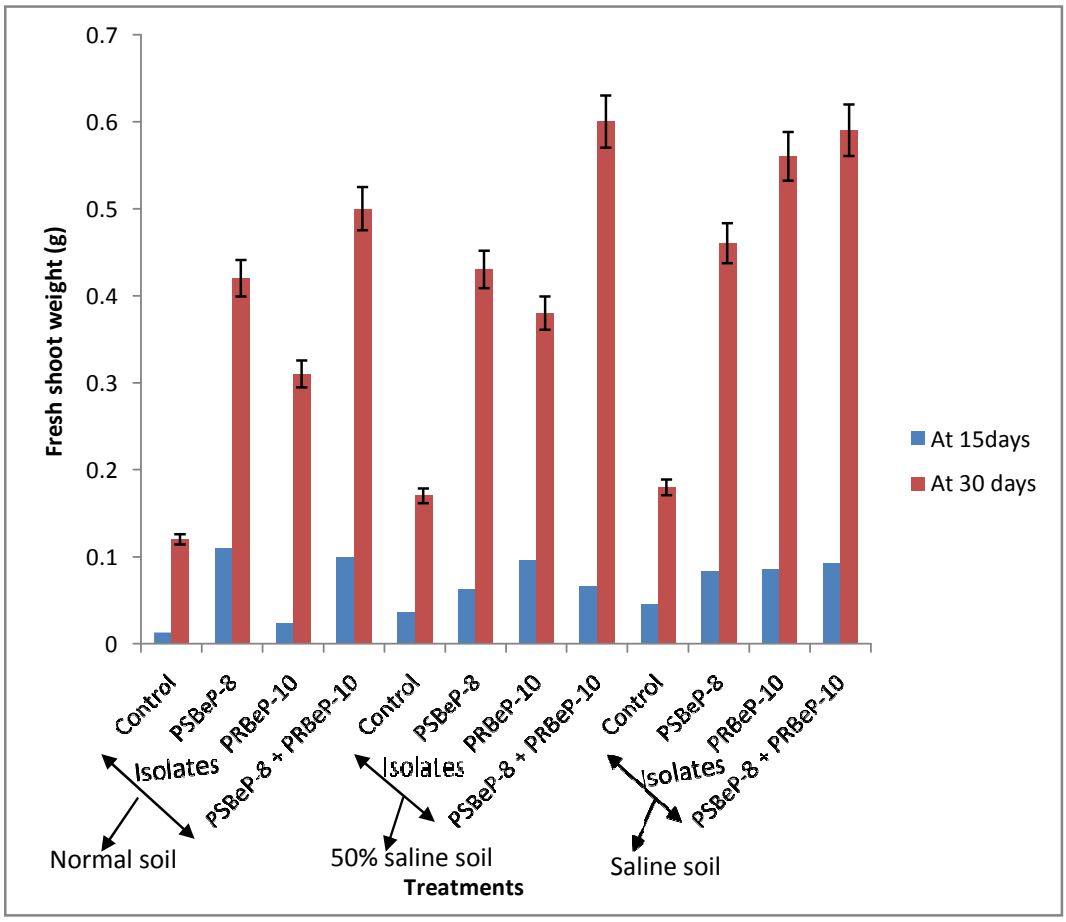


Fig.4.9.Influence of bacterial inoculation on fresh shoot weight of fenugreek plants at 15 and 30 days after germination.

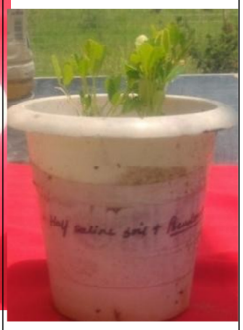
4.11 Identification and characterization of potential bacterial endophytes

Two selected endophytic bacterial isolates (PSBeP-8 and PRBeP-10) were characterized and identified using morphological, biochemical and molecular markers.

Both the bacterial endophytes were grown on nutrient agar plates and nutrient broth and active cultures were evaluated for various morphological and biochemical traits. The young discrete colonies appearing on nutrient agar were visualized for texture, margin, elevation and chromo genesis. The isolate PSBeP-8 showed small flat colonies with smooth surface and entire edge and brownish colour green fluorescence while the isolate PRBeP-10 showed smooth, round colonies with entire edge and greenish yellow colour. Microscopic observations reveal that both the bacteria were having short rods with single occurrence. Both the cultures were found to be gram negative and non-Sproulating bacteria (Table.4.13.a). The active broth cultures of both the bacterial endophytes were also evaluated for various biochemical features as shown in Table.4.13.b.Both the cultures showed positive test for oxidase production, catalase production, gelatin hydrolysis, siderophore production, phosphatase production, salt stress tolerance, alkaline stress tolerance. Both the cultures were negative for methyl red, urease production test and HCN production.(Table.4.13.b)

After evaluating morphological and biochemical characteristics, both the cultures were further identified using molecular markers. The genomic DNA was extracted from both the cultures as shown in plate 4.4.a. DNA samples were sent to M/s chromous biotech private limited, Bangalore for 16S rRNA gene sequence analysis. The PCR amplified product (16S rRNA gene amplicon) of both the bacterial isolates are shown in plate 4.4.b.The 16S rRNA sequences were retrived from NCBI database and aligned with the 16S rRNA sequences of two bacterial isolates. The 16S rRNA gene from the isolate PSBeP-8 showed 99% similarity with *Pseudomonas aeruginosa* (Table 4.14.a).Phylogenetic tree was constructed by neighbor joining method using the software MEGA 7.0 and on tree also it showed close relatadness with *Pseudomonas aeruginosa*.(Fig 4.10.a).The 16S rRNA gene from the isolate PRBeP-10 showed maximum similarity (99%) with an unidentified species of *Sphingobacterium* (Table 4.14 b).Phylogenetic tree was constructed by neighbor joining method using the software MEGA 7.0. and on tree also it showed close relatadness with *Sphingobacterium* sp(Fig 4.10.b). To the best of our knowledge, *Sphinobacterium* sp as an endophyte of the medicinal weed plant, *Phyllanthus* sp, is being

Plate 4.2: Influence of inoculation of mono-cultures and their co-cultures on growth of leafy vegetable fenugreek under pot culture experiment using soil with different levels of salinity

Soil condition	Control	Isolate PSBeP-8	Isolate-PSBeP-10	Coinoculation (PSBeP-+PRBeP-10
Normal soil				
Half-saline soil				
Saline soil				

reported for the first time. Since Phyllanthus the medicinal weed naturally grows in local areas and it is also grown in underutilized regions without any input of agrochemicals, therefore it seems that its association with endophytic bacteria is mainly responsible for the dominant existence of this weed plant. Therefore dominant existence of sphinobacterium as endophytes in medicinal plants is of significant importance in growth and development of plants in disturbed soils. According to most of the earlier reports on endophytic bacteria isolated from medicinal plants belong mainly to the genus *Pseudomonas* and *Bacillus*.

(a) **(b)**

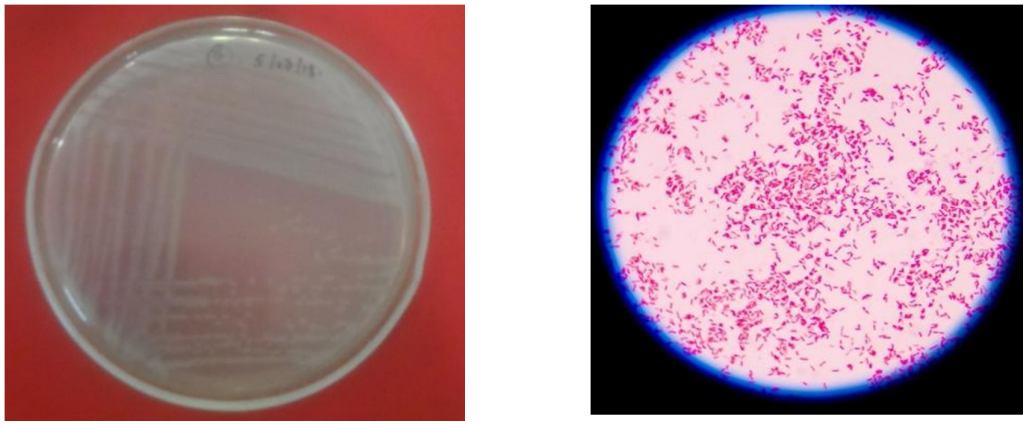


Plate.4.3. Colony morphology (a) and photomicrograph (b) of the selected halo-alkalo tolerant endophytic bacterial isolate PSBeP- 8.

Table 4.13(a). Morphological characteristics of the two halo-alkalo tolerant endophytic bacterial isolates (PSBeP- 8 and PRBeP- 10) used in pot culture study.

Bacterial Isolates	Colony morphology	Cellular characteristics			Sproulation	Gram's reaction
		Cell size	Shape	Arrangements of cells		
PSBeP-8	Small, flat colonies, smooth surface entire edge, brownish color with greenish fluorescence	0.5-1.0µm in diameter	Short rods	Single	Negative	Negative
PRBeP-10	Smooth, round and greenish yellow colonies with entire edge	0.6-0.7µm	Short rods	Single	Negative	Negative

Table 4.13(b). Biochemical characteristics of the two halo-alkalo tolerant endophytic bacterial isolates (PSBeP- 8 and PRBeP- 10) used in pot culture study.

S.No	Biochemical parameters	Isolates showing results(+/-)	
		PSBeP-8	PRBeP-10
1	Oxidase production	+	+
2	Catalase production	+	+
3	Metthyl red test	-	-
4	Urease production	-	-
5	Gelatin hydrolysis	+	+
6	Siderophore production	+	+
7	Phosphatase production	+	+
8	HCN production	-	-
9	IAA production	+	+
10	Ammonia production	-	-

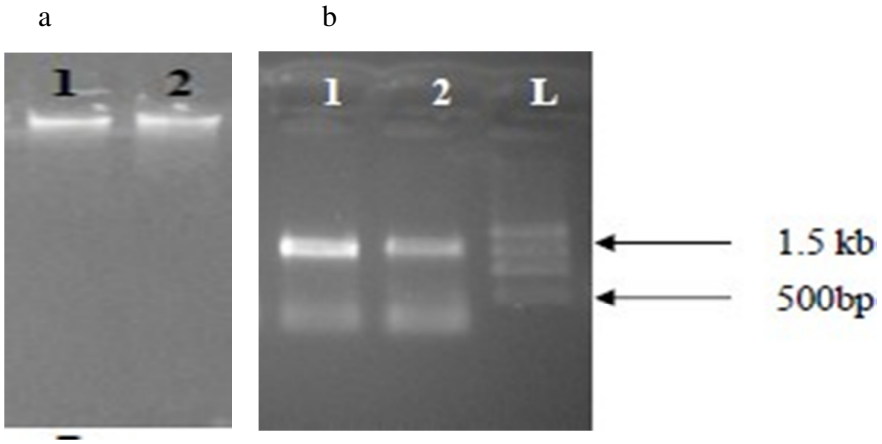


Plate 4.4.a: Genomic DNA from bacterial isolates PSBeP-8 and PRBeP-10 Lane 1: PSBeP-8, Lane 2: PRBeP-10

Plate 4.4.b. PCR amplified products of 16S rRNA gene segment from the bacterial isolates PSBeP-8 and PRBeP-10: Lane 1: PSBeP-8, Lane 2: PRBeP-10

Table 4.14 a.Percent identity of the bacterial isolate PSBeP-8 with other *Pseudomonas* cultures based on 16S rRNA gene sequence analysis.

Sl. No.	*Organism Name*	Accession No.	Percentage Match
1	*Pseudomonas sp. Pseudo-EJB5*	GU966670.1	99%
2	*Pseudomonas aeruginosa isolate 9*	FJ907192.1	99%
3	*Uncultured bacterium clone PLB97*	KT285549.1	99%
4	*Pseudomonas sp. AGP-01*	HM587311.1	99%
5	*Pseudomonas aeruginosa NO5*	FJ972533.1	99%
6	*Pseudomonas aeruginosa strain D1*	KF113578.1	99%
7	*Pseudomonas aeruginosa strain SUB 10*	KC920813.1	99%
8	*Bacterium H1C*	JX149543.1	99%
9	*Pseudomonas aeruginosa strain HS9*	GU323371.1	99%
10	*Bacillus sp. W4(2008)*	EU596423.1	99%

Table4.14a .Percent similarality of the bacterial isolate PRBeP-10 with Sphingo bactirum sp based on 16S rRNA gene sequence analysis.

Sl. No.	*Organism Name*	Accession No.	Percentage Match
1	*Sphingobacterium sp. MIMdw12*	KT347098.1	99%
2	*Sphingobacterium spiritivorum strain HAMBI 1896*	LT899987.1	97%
3	*Sphingobacterium spiritivorum strain S8CF13*	KP326564.1	97%
4	*Uncultured bacterium clone 10*	KP003913.1	97%
5	*Sphingobacterium spiritivorum strain: NBRC 14975*	AB680729.1	97%
6	*Sphingobacterium spiritivorum strain NBRC 14948*	NR_113707.1	97%
7	*Sphingobacterium spiritivorum strain BIHB 346*	FJ859692.1	97%
8	*Sphingobacterium spiritivorum strain NCTC 11386*	NR_044077.1	97%
9	*Sphingobacterium spiritivorum strain: JCM 1733*	LC060921.1	97%
10	*Sphingobacterium strain: JCM 1732*	LC060920.1	97%

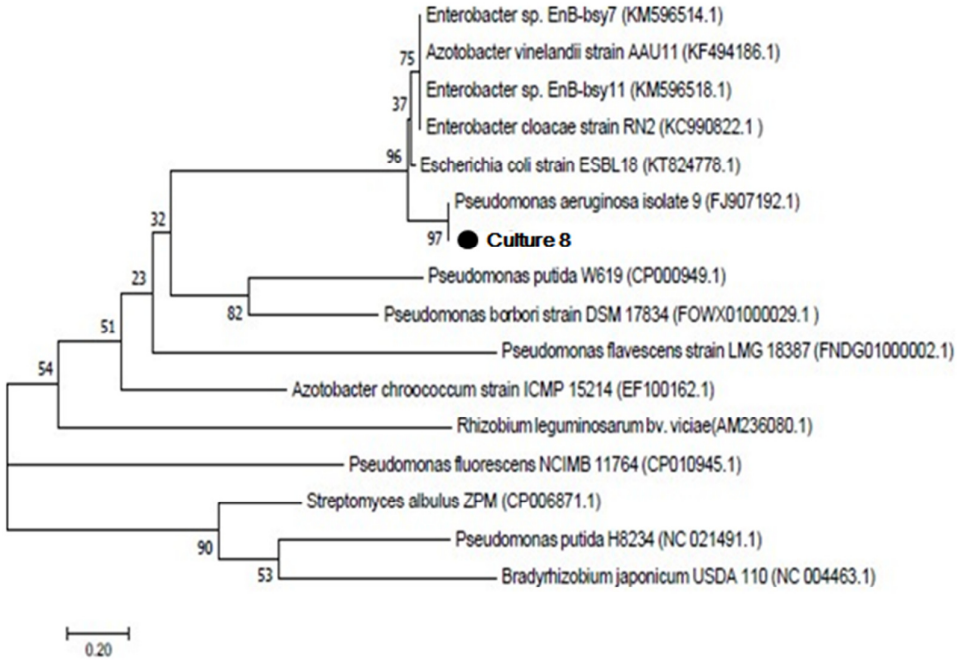


Fig 4.10 (a) Phylogentic tree showing its genetic relatedness of the bacterial endophyte PSBeP- 8 showing with other members of the genus Pseudomonas aeruginosa, constructed using 16s rRNA gene sequence retrived from the data base using neighborhood joining method. The bootstrap values were generated from 1000 replicates.

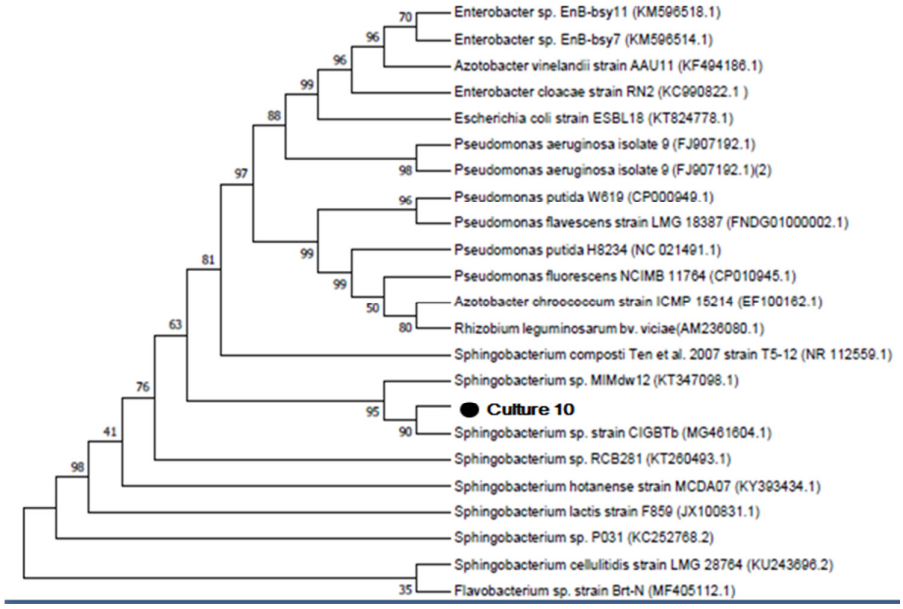


Fig 4.10 (b) Phylogentic tree showing its genetic relatedness of the bacterial isolate PRBeP-10 with other members of the genus *Sphinobacterium sp* constructed using 16S rRNA gene sequences retrived from the data base using neighbor joining method. The bootstrap values were generated from 1000 replicates.

Summary and Conclusions

Salt-affected soils are becoming a great threat to agricultural production worldwide. Recently such area is increasing at an alarming rate also hindering the vegetable production worldwide. The underutilized medicinal plant, *Phyllanthus* sp has been reported as important source of endophytic microorganisms, which are capable of improving the plant growth under various stressful conditions. In this regard, the present study was focused on the isolation of endophytic bacteria from various parts of *Phyllanthus* sp, identification of their in vitro salt/alkaline pH tolerance, PGP traits and in vivo evaluation of their efficacy to enhance the leafy vegetable biomass of fenugreek under pot culture conditions.

1. A total of 27 endophytic bacteria were isolated from different parts stem/root of *Phyllanthus* sp. and screened for their *in vitro* abiotic stress tolerance and designated as PSBeP (1-11) for stem endophytes, PRBeP (1-10) for root endophytes

2. The bacterial endophytes were screened qualitatively for their alkaline pH tolerance(using nutrient agar adjusted with pH 7-11) based on their abilities to grow under such conditions and only eleven isolates showing growth in pH range of 9.0-10.0 were selected, none of the isolate could grow at pH 11.0.

3. The eleven alkalo-tolerant endophytic isolates were further screened for their salt tolerance. All the isolates exhibited growth upto 6 % NaCl concentration, only the isolate PRBeP-6 could grow at 10% NaCl concentration and no growth was recorded at 12% NaCl in any case.

4. The bacterial isolates were screened for showed for in vitro plant growth abilities. All the isolates showed P-solublization and gelatin hydrolysis while varied potential for other PGP traits. All the isolates were then compared for salt tolerance, alkaline pH stress tolerance, plant growth tarits on the basis of which four potential isolates (PRBeP-6, PRBeP-10, PSBeP-8 and PSBeP-11) were selected for further studies

5. The four selected bacterial isolates were evaluated for their growth potentialities under abiotic stress conditions (pH 7.0-10.0) and growth curves were drawn. All the isolates invariably exhibited maximum growth at normal pH (7.0) within 24 h. The only bacterial

isolate (PRBeP-10) exhibited maximum alkali tolerance up to pH 10.0,while rest of the isolates PRBeP-6, PSBeP-8,PSBeP-11 exhibited growth up to pH 9.0.All the isolates were then evaluated for salt tolerance limits, in which isolate PRBeP-6 ,PRBeP-10, PSBeP-8,PSBeP-11 exhibited growth upto 10% NaCl concentration.

6. The isolates were screened for their in vitro salt tolerance limits using varying concentrations of NaCl (0-12%). With increasing salt concentration, the growth rate of the bacteria also decreased along with an elongated lag phase. None of the isolate showed growth at 12% NaCl concentration while at 10% NaCl concentration, the bacterial isolate PSBeP-8 and PRBeP-10 showed better growth in comparison to other two isolates.

7. The four bacterial endophytes were screened for their *in vitro* phosphate solubilizing potential using mineral salt medium amended with TCP as the only phosphorous under normal conditions of pH 7.0 and salt concentration (0% NaCl) among the bacterial endophytes, the isolate PRBeP-6 exhibited highest activity showing 269.27 µg/mL phosphate in the culture filtrate at 72 h which was followed by the isolate PSBeP-8 showing 223.52 µg/mL phosphate in the culture filtrate at same time period. P-solubilizing potential of the isolates was also determined under abiotic stress conditions i.e. at wide pH range of 7.0-10.0 and salt (NaCl)concentrations of 0-10%.Among the isolates, the isolate PRBeP-10 exhibited highest P-solublization at highly alkaline condition (pH 10.0) with phosphate concentration of 61.18 µg/mL at72 h. At pH 11.0 no phosphate was observed in the culture filtrate in any case. Under saline stress conditions, the selected isolates exhibited varying p-solublization with no solubilized phosphate in culture filtrate at salt concentration 12%, the isolate PRBeP-6 displayed good activity initially at 0% NaCl its P-solublization activity decreased increasing salt concentration, while the endophytic cultures PSBeP-8 and PRBeP-10 exhibited consistent P- solublization activity with phosphate concentration of 34.15µg/mL and 38.47µg/mL respectively in their culture at 10% NaCl concentration in 72 h the values being significantly higher than those for other cultures. .

8. The four selected endophytes were also screened for their siderophore production under normal conditions (pH-7, Nacl-0%) and under alkaline pH stress (pH 7-10) and salt stress(0-10% NaCl) conditions the siderophore production was quantified spectrophotometrically in the culture filtrates up to 96 h. Under normal conditions, all the cultures showed siderophore production potential, but the isolate PSBeP-11 exhibited

highest siderophore(sid) units (91.71 %).However, the isolates PRBeP-10 and PSBeP-8 retained siderophore producing capacity even under the high alkaline pH of 10.0 showing 46.58% and 44.37% sid units respectively at 72 h.

Under salt stress conditions, the isolates varied considerably in their siderophore producing capabilities showing reduced sid units at higher salt concentrations. Isolate PSBeP-8 exhibited highest siderophore production with 28.33 % sid units at 10 % NaCl while isolate PRBeP-10 exhibited 21.77% sid units at the same NaCl concentration at 72 h.

10. After intensive qualitative and quantitative testing of the four selected bacterial endophytes for stress tolerance, P-solublization, siderophore production and various plant growth promoting traits, two bacterial isolates PRBeP-10 and PSBeP-8 were selected and evaluated for their influence on *in vitro* seed germination of fenugreek, under normal and abiotic(pH/salt) stress conditions. The isolate PRBeP-10 increased the germination of fenugreek seeds to maximum extent (98.67%) compared to uninoculated seeds under alkaline pH condition (pH-10) at72 h. Under salt stress conditions, the isolates PRBeP-10 and PSBeP-8 increased the germination rate of fenugreek seeds at the same rate.

11. *In vivo* pot culture experiments were carried out using saline/alkaline soil (pH 9.0) for evaluating the efficacy of two potential isolates(PRBeP-10 and PSBeP-8) and their co-cultures on leafy biomass yield of leafy vegetable fenugreek(*Trigonella foenum-graceum*) under three levels of soil salinity(normal soil, half saline soil and saline soil). Higher leafy biomass(fresh shoot weight) was recorded for the fenugreek plants inoculated with the monocultures of the two isolates (PRBeP-10 and PSBeP-8) as compared to the uninoculated plants. However, the co-inoculation with two isolates (PRBeP-10+PSBeP-8) further enhanced the rate of seed germination as well as plant growth and fresh weight of shoots as compared to uninoculated or mono-culture treated plants at all the three levels of soil salinity. But the results were more significant under saline soil conditions after 30 d of germination compared to uninoculated/mono-cultured inoculated plants.

The underutilized medicinal weed, *Phyllanthus* sp was identified as rich source of plant growth promoting endophytic bacteria equipped with several additional potentialities such as salt/alkalinity stress tolerance, nutrient solubilizing and siderophore producing potential under abiotic stress conditions.The bacterial endophytes(PSBeP-8 and PRBeP-10) from *Phyllanthus* sp were identified as most halo-alkalo-tolerant strains, which were

identified as *Pseudomonas aeruginosa* PSBeP-8 and *Sphingobacterium* sp PRBeP-10 based 16S rRNA gene sequence analysis. *In vivo* studies using saline soil (pH 9.0) indicated enhancement of seed germination and leafy biomass yield of the leafy vegetable fenugreek under saline soil conditions. . Hence the bacterial endophytes PSBeP-8 and PRBeP-10 emerged as a potential halo-alkalo strains, which can mitigate the harmful effects of salt stress in agricultural soils.

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ABSTRACT

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			Department	:	Microbiology
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Salt- affected soils are becoming greatest hindrance to crop production worldwide; the rate at which the arable land is lost every year due to these problems is imposing a greatest challenge to agricultural production. Hence in order to ameliorate the stress imposed by these soils on the crop plants, microbial mediated approach is selected and the present study is conducted to address this issue. Twenty seven indigenous endophytic bacteria were isolated from underutilized medicinal weed plant *Phyllanthus* sp and screened for *in vitro* pH (7.0-11.0) stress tolerance. Eleven alkalo-tolerant isolates were selected and further screened for in vitro salt tolerance(using NaCl concentration 0-12%) and plant growth promoting traits. The four superior isolates(PSBeP-8,PSBeP-11) showing higher stress tolerance and PGP properties were selected. The isolate PRBeP-10 was found to be most akalo-tolerant showing growth up to pH 10.0 while the isolate PSBeP-8 most salt tolerant with growth up to 10% NaCl concentration. Under normal conditions (pH 7.0;NaCl 0%) all the isolates showed P- solublization and siderophore production which was adversely affected with increasing salt and pH conditions. The isolate PRBeP-10 showed higher phosphate solublization under pH/salt stress conditions as compared to other cultures showing phosphate concentration of 61.18 µg/mL at pH 10.0 and 38.4 µg/mL at 10% NaCl concentration. Siderophore production at highly alkaline pH(pH 10.0) was shown by the isolate PRBeP-10(46.58% sid units in 72 h while at highly saline condition(10% NaCl) 28.3% sid units were recorded for PSBeP-8 in the same time. Thus the isolates PSBeP-8 and PRBeP-10 were found most halo-alkalo-tolerant retaining their p-solubilizing and siderophore producing abilities under pH and salt stress conditions. Both the isolates were evaluated for their *in vitro* and *in vivo* growth enhancing capabilities under normal and saline/alkaline conditions. Germination of fenugreek seeds was increased under salt and pH stress conditions due to inoculation with bacterial endophytes. During pot culture experiment, the mono-cultures of PRBeP-10 and PSBeP-8 and their co- culture inoculation enhanced the leafy biomass yield of fenugreek plants over their corresponding un treated controls grown in saline soil(of pH 9.0).Based on various morphological, bio-chemical traits and 16S rRNA gene sequence analysis, the isolate PRBeP-10 and PSBeP-8 were identified as *Sphingobacterium* sp PRBeP-10 and *Pseudomonas aeruginosa* PSBeP-8.

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षोध षीर्षक	:	" खारे तनाव की स्थिति के तहत ट्रिगोनेला फीनम–ग्रेक्यूम (मेथी) की वृद्धि और तनाव सहनशीलता के लिए एंडोफेटिक बैक्टीरिया का मूल्यांकन"			
सलाहकार	:	डा0 लक्ष्मी तिवारी			

दुनिया भर में फसल उत्पादन के लिए नमक–प्रभावित मिट्टी सबसे बड़ी बाधा बन रही है। इन समस्याओं के कारण हर साल जिस दर से कृषि भूमि लुप्त हो रही है वह कृषि उत्पादन के लिए सबसे बड़ी चुनौती बन रहा है। इसलिए फसल के पौधों पर इन मिट्टी द्वारा लगाए गए तनाव को कम करने के लिए, जैविक मध्यस्थ दृष्टिको ा का चयन किया जाता है और वर्तमान अध्ययन इस मुद्दे को हल करने के लिए किया जाता है। 20 स्वदेशी एंडोफिटिक बैक्टीरिया को औषधीय खरपतवार संयंत्र फिलांथस एसपी से पृथक किया गया था और इन विट्रो पीएच (7.0–11.0) तनाव सहनशीलता के लिए जाँच किया गया था। 11 एल्कोलो–सहिष्णु पृथक का चयन किया गया और इन विट्रो नमक सहनशीलता (0–12 : छंब्स का उपयोग करके) और लक्षणों को बढ़ावा देने के लिए पौधों की वृद्धि के लिए आगे की जांच की गई। चार बेहतर पृथक (पी0 एस0बीई0 पी0 – 8, पी0 एस0 बीई0 पी0 –11) उच्च तनाव सहनशीलता दिखाते हैं और पी0 जी0 पी0 गु ाों का चयन किया गया था। पृथक पी0 आर0 बीई0 पी –10 पीएच 10.0 तक विकास को दिखाते हुए सबसे अधिक एकेलो–सहिष्णु पाया गया था, जबकि पृथक पी0 एस0 बीई0 पी0 – 8 विकास के साथ 10: छंब्स सांद्रता में सबसे अधिक नमक सहनशील था। सामान्य परिस्थितियों में (पीएच 7.0 तथा छंब्स 0:) सभी पृथक ने पी–घुलनशीलता और सीडेरोफोर उत्पादन दिखाया जो कि नमक और पीएच की स्थिति में वृद्धि के साथ प्रतिकूल रूप से प्रभावित था। पृथक पी0 आर0 बीई0 पी0 –10 ने पीएच /नमक तनाव की स्थिति के तहत पीएच/नमक तनाव की स्थिति के तहत उच्च फॉस्फेट घुलनशीलता दिखाया, जबकि अन्य संस्कृतियों की तुलना में पीएच 10.0 और 38.4 माइक्रोग्राम/ एमएल पर फॉस्फेट एकाग्रता 61. 18 माइक्रोग्राम/एमएल 10: सांद्रता पर दिखाती है। अत्यधिक क्षारीय पीएच (पीएच 10.0) पर साइडरोफोर उत्पादन अलग पी0 आर0 बीई0 पी0 –10 द्वारा दिखाया गया था (72.58: एसआईडी इकाइयों 72 एच में जबकि अत्यधिक नमकीन स्थिति (10: छंब्स) पर 28.3: सिड इकाइयों को पी0 एस0 बीई0 पी0 –8 के लिए एक ही समय में दर्ज किया गया था इस प्रकार पी0 एस0 बीई0 पी0 –8 और पी0 आर0 बीई0 पी0 –10 को अलग–अलग हेलो–एल्कोलो–सहिष्णु पीएच और नमक तनाव की स्थिति के तहत अपने पी–घुलनशीलता और सीडेरोफोर उत्पादन क्षमताओं को बनाए रखते थे। दोनों पृथकों का मूल्यांकन उनके इन विट्रो और विवो विकास बढ़ाने क्षमताओं में किया गया था सामान्य और नमकीन/क्षारीय स्थितियों के तहत। मेथी के बीज के अंकुर ा को बैक्टीरियल एंडोफाईट्स के साथ इनोक्यूलेशन के कार ा नमक और पीएच तनाव की स्थिति में वृद्धि हुई थी। पॉट संस्कृति प्रयोग के दौरान, पी0 आर0 बीई0 पी0 –10 और पी0 एस0 बीई0 पी0 –8 की मोनो–संस्कृतियां और उनके सह–संस्कृति इनोक्यूलेशन नमकीन मिट्टी (पीएच 9.0 के) में उगाए गए उनके संबंधित गैर–नियंत्रित नियंत्र ाों पर मेथी के पौधों की पत्तेदार बायोमास उपज में वृद्धि हुई। विभिन्न रूपात्मक, जैव–रासायनिक लक्ष ाों और 16 एस आर0 एन0 ए0 जीन अनुक्रम विश्लेषण पर आधारित, पृथक से पी0 आर0 बीई0 पी0 –10 और पी0 एस0 बीई0 पी0 –8 को *स्पिंगोबोबैक्टेरियम* एसपी पीआरबीईपी –10 और *स्यूडोमोनास एरुजिनोसा* पीएसबीईपी –8 के रूप में पहचाना गया था।

(लक्ष्मी तिवारी)
सलाहकार

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लेखक