Isolation and Molecular Identification of Salt Tolerant Probiotic Bacteria and their Impacts on Rice (*Oryza sativa*) Seedlings

By

Mahmud -Ur- Rahman
Student ID: 18376009

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Mahmud -Ur- Rahman

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Mahmud -Ur- Rahman
Student ID: 18376009

Approval

The thesis/project titled "Isolation and Molecular Identification of Salt Tolerant Probiotic Bacteria and their Impacts on Rice (*Oryza sativa*) Seedlings" submitted by Mahmud -Ur-Rahman (Student ID 18376009) of Fall, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science on 23rd October, 2021.

Examining Committee:

Supervisor:
(Member)

_____

Professor Dr. Md Tofazzal Islam
Founding Director and Head, Plant Biotechnology Discipline, IBGE, BSMRAU, Gazipur

Internal Supervisor:
(Member)

_____

Dr. Iftekhar Bin Naser
Associate Professor, Department of Mathematics and Natural sciences, BRAC University, Dhaka

Program Coordinator:
(Member)

_____

Dr. Iftekhar Bin Naser
Associate Professor, Department of Mathematics and Natural sciences, BRAC University, Dhaka

Departmental Head:
(Chair)

_____

Professor A F M Yusuf Haider, PhD
Professor and Chairperson, Department of Mathematics and Natural Sciences, BRAC University, Dhaka

ABSTRACT

Soil salinity is a serious problem in the coastal areas of Bangladesh. It is the main barrier to attain sustainability in crop production in those particular areas. Moreover, the situation is getting worse along with time. Therefore, an eco-friendly and sustainable approach is required to overcome this problem. Discovery of plant growth promoting bacteria (PGPB) and their application to crop plants are considered as promising and effective biotechnological approaches to fight against salt-stress to crop plants. The objectives of this study were to isolate and characterize salt tolerant PGPB from the rice plants cultivated in saline soils and evaluate their performances on seed germination and seedling growth of rice. The study was performed at the laboratories of the Institution of Biotechnology and Genetic Engineering (IBGE) in Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU). Rice plant samples were collected from salt-affected areas in Chattogram, Noakhali, Lakshmipur and Cox's Bazar districts. Forty one salinity tolerant bacteria were isolated and characterized for screening *in vitro* for both salinity tolerance, and three highly salt tolerant isolates were further evaluated on seed germination and seedling growth of rice cv. BRRI dhan 29 (salinity susceptible) and BINA dhan 10 (salinity tolerant). Priming of rice seeds with three highly salt tolerant (up to 12% NaCl w/v) isolate viz. BTCoSo2, BTCoR2 and IBGE3C promoting growth of rice seedling and the effects were pronounced in BINA dhan 10. Among the three bacteria, IBGE3C displayed best performances on seedling growth of rice under varying salinity was sequenced and the strain exhibited 100% 16S ribosomal RNA gene sequence homology with *Brevibacterium sediminis* IBGE3C (accession no. MZ573246) strain. *Brevibacterium sediminis* is a potential plant growth promoting bacteria and it can significantly increase the shoot and root length in plants. Also, the isolation of *B. sediminis* from sea water and deep-sea sediments has been previously reported

which suggests it as a potential salt tolerant bacteria. *Brevibacterium* spp. are rod- shaped, non-spore forming gram-positive bacteria. This study for the first time identified *B. sediminis* strain IBGE3C (accession no. MZ573246) as a salt tolerant PGPB from the rice cultivated in Lakshmipur district of Bangladesh. In addition, the collected rice variety was BRRI dhan 28 which is also a widely cultivated variety throughout the country. However, the bacteria has been isolated from plant's root sample which is required for further study for its practical application in the enhancement rice production in the saline soils in Southern districts of Bangladesh.

Keywords: Soil salinity, Probiotic, Salinity stress, Plant growth promotion

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Sincerely,

Mahmud -Ur- Rahman

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List of Acronyms

IBGE	Institute of Biotechnology & Genetic Engineering
BSMRAU	Bangabandhu Sheikh Mujibur Rahman Agricultural University
PGPR	Plant Growth Promoting Rhizobacteria
PGPB	Plant Growth Promoting Bacteria
EC	Electrical Conductivity
ST	Salt Tolerant
ACC	1-Aminocyclopropane-1-carboxylic acid
IAA	Indole-3-Acetic Acid
IPT	Isopentyl Transferase
Ck	Cytokines
GA	Gibberellic Acid
ABA	Abscisic Acid
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
CAT	Catalase
POD	Peroxidase
APX	Ascorbate Peroxidase
DHAR	Dehydroascorbate reductase
MDAR	Monodehydroascorbate reductase
GPX	Glutathione Peroxidase
GST	Glutathione s-Transferase

CHAPTER I

INTRODUCTION

Many crop plants are sensitive to high salt concentration that inhibits the productivity of plants. Salinity is a serious environmental hazard for plants and it is getting worse day by day because of global climate change. Especially in the coastal areas, farmers struggle a lot against salinity for the production of their crops. Also, this threat can bring food scarcity in Asian subcontinent where the major crops are rice (*Oryza sativa*) and wheat (*Triticum aestivum*). The coastal zone occupied 20% of the total land along with covering 30% of the total cultivable land in Bangladesh (Hasan et al., 2019). However, scientists are working to cope up with the problems of salinity and their strategies include resource management, developing better breeds and even transgenics. Since all these strategies are lengthy and cost intensive, there is a scope of more research on developing a sustainable and cost effective approach. In this regard, the role of microorganisms, especially plant growth promoting rhizobacteria (PGPR), is very effective and significant and there is evidence that these PGPRs have potential to decrease salt stress hazards in plants. Water and soil resources are the main aspects of agricultural practice, but the 21st century starts with global water scarcity and salinization of soil and water. Current world is struggling to establish sustainable development in agriculture because of increasing population thereby decreasing cultivable land. When the electrical conductivity (EC) of the saturation extract (ECe) in the root zone passes 4 dS m^{-1} (about 40 mM NaCl) at 25º and has an exchangeable sodium of 15%, then the soil is called saline soil. Jamil et al., (2011) reported that more than half of the total cultivable land would be saline affected by the year 2050. In addition, many reasons viz. irrigation with saline water and poor cultural practices are increasing the amount of salinized soil at a rate of 10% every year. Along with decreasing yield, salt stress also

alters the quality of crops. Salinity strikes plants in various ways including abnormalities in flowering and fruiting pattern, delayed formation of roots and shoots etc. Although plants have self defense mechanisms, they cannot cope up with severe salinity. Therefore, additional approaches such as inoculating plants with PGPRs offers a great advantage to combat salt stress. PGPB have remarkable impacts to ensure normal growth and development of plants under salinity and so far many bacterial strains viz. Pseudomonas spp, Frankia spp, Rhizobia spp have been identified that are capable of aiding plants under several environmental stresses (Glick, 2012). Also, the magnificent benefits of PGPB has developed a large trading platform; the commercialization of PGPB has been reported in Vessey (2003); Lucy et al. (2004).

Aim of the Research

Rice (*Oryza sativa*) is the most extensively cultivated and staple food crop in Bangladesh. Salinity tolerant bacteria associated with rice plants may be exploited as natural bioagents for the enhancement of growth and yield of rice in salinity affected areas. Therefore, the goal of the current study was to identify and characterize salinity tolerant rice-associated probiotic bacteria and evaluate their effects on growth and salinity tolerance in rice seedlings.

To obtain this aim, the particular objectives of the present research were to:

1. Isolate and screen salinity tolerant probiotic bacteria from different organs of rice plant cultivated in saline soils.
2. Assess the effects of salt tolerant probiotic bacteria on growth of rice seedlings.
3. Identify the most effective salt tolerant probiotic bacteria using 16S rRNA gene sequencing.

CHAPTER II

LITERATURE REVIEW

2.1 Problems of salinity

Around 800 million hectares of cultivable land has been affected by soil salinity throughout the entire world (Munns & Tester, 2002). The problems and the effects offer different scenarios in different types of land and these limit the plant's normal outcome (Ashraf & Harris, 2014). For example, the irrigation system in desert areas is responsible for the accumulation of salt in the rhizosphere of plants which eventually affects the plant and the land (Qadir et al., 2014; Hussain et al., 2009). However, the consequence of salinity in land affects both the production of crops as well as employment. Studies that were performed in India show that soil salinity decreases the production of rice, wheat, cotton and sugarcane by 45%, 40%, 63%, 68% respectively (Tripathi, 2009). Plants are being harmed by soil salinity in various ways as the phenomenon results in abnormalities in physiology especially in the pattern of flowering and fruiting.

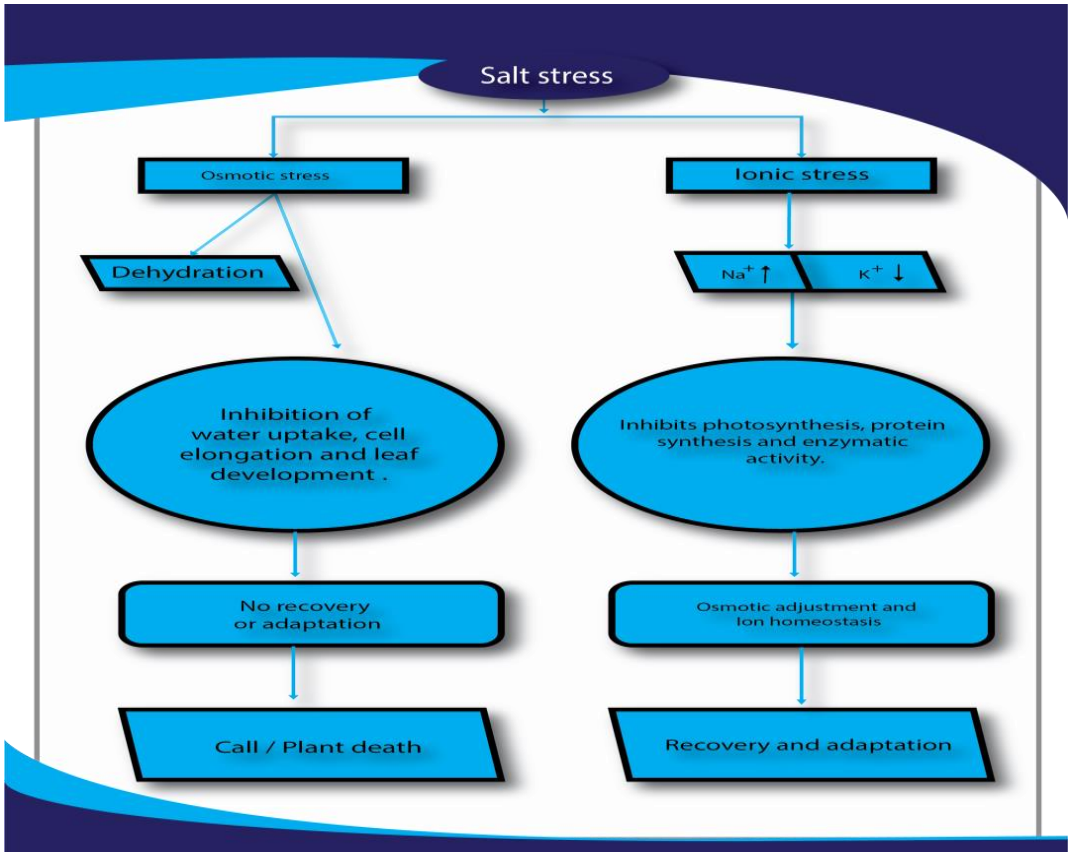


Figure1: Consequences of salt stress in plants

Also, it has adverse impacts on the production and the biomass. Study shows that root and shoot formation occur slowly and the flowering transition time is also affected by high salt concentration (150 mm NaCl) in tomatoes (Ghanem at el., 2009). A similar phenomenon has also shown in chickpea due to the high level of Na+ in the thin layers of expanded leaves (Pushpavalli et al., 2016).

2.2. Mechanism of salt stress management in plants

Plants adjust themselves to cope up with salinity in soil and the changes include physiological, morphological and biological. Basically, the main action of a plant's defense system against salt

stress involves limiting the loss of water to keep the photosynthesis process intact (Acosta et al., 2017). Plants are usually divided into two major groups (glycophytes and euhalophytes) and their reaction varies regarding different factors such as osmotic regulation, electron transport, toxic ion accumulation, CO_2 assimilation, chlorophyll content, antioxidant defense and reactive oxygen species generation (Tang et al., 2015; Munns, 2005; Koyro, 2006; Stepien & Johnson, 2009). However, most crop plants fall in the glycophytes group and they cannot tolerate high salt concentration and consequence is the death of plants (Hernández & Almansa, 2002). On the contrary, since the halophytes hold better protection to salinity they can tolerate high salt concentration (300-500 mM) (Parida & Das, 2005; Flowers & Colmer, 2015). Moreover, they usually balance their salt content through several ways such as salt exclusion, salt elimination and salt succulence (Acosta et al., 2017)

2.2.1 Morphological adjustment

Munns (2005) reported that salinity does not directly affect the growth of plants, but it affects the photosynthesis process and the function of particular catalysts as well. The author actually talked about morphological adaptations of plants in response to salinity and he developed a representation that shows the salt-oriented impacts on plant growth in two-phase. In his model, growth is first inhibited by a reduction in the soil water potential and then a particular effect seems as salt injury in leaves. Moreover, these leaves eventually die as the vacuoles are no longer able to separate the incoming salt which gets accumulated in the cell walls or cytoplasm. He adds that this phenomenon inhibits the growth by decreasing the supply of growth hormones to the meristematic region.

Besides these, accumulation of high amounts of salt is directly related to the decrease of photosynthesis rate and the synthesis of particular metabolites that suppress development of plants (Azza et al., 2007).

Moving on to the root morphology, the accumulation of water and nutrients in plants and the compensation of plant water loss is governed by the characteristics of the root system such as root diameter, root length etc (Passioura, 1988). Also, environmental aspects like salinity can generate a remarkable impact on root anatomy and the consequence is thickened and complicated cell walls (Shannon et al., 1994).

2.2.2. Contribution of potassium

Potassium ion is the most essential nutrient in plant cells which is crucial for many important enzymatic reactions. Also, it is important for ionic and pH homeostasis and it maintains adequate membrane potential (Maathuis, 2009; Ahmad and Maathuis, 2014). Moreover, cytosolic potassium is an important detector of a plant's adaptive reaction mechanism to a large spectrum of environmental stresses (Shabala & Pottosin, 2014). Besides these, potassium ions have more importance than sodium ions in many biochemical and physiological roles. In addition, salinity induced impacts on potassium transport depends on variety, tissue and cell. For instance, salt tolerant barley varieties are able to hold more potassium ions in their roots (Chen et al., 2007). However, several study suggest that potassium ion have significant role in cell signaling under salt stress and the potassium ion holding capability is an important characteristics of plants to salinity (Shabala 2009, 2017; Anschütz et al., 2014; Shabala and Pottosin, 2014; Wu et al., 2018)

2.2.3. Function of vacuole

Plants restrict the flow of salt from cytoplasm to leaf by two mechanisms whereas salt ions can get assembled either in the apoplast or move to the vacuole. Because of building up salt ions in the apoplast, the osmotic gradient between inside and outside of the cell increases automatically. Eventually, this fact causes cell death as the cells get dehydrated due to the diffusion of water from the inside of the cell to intracellular spaces to adjust a thermodynamic equilibrium. For this reason, accumulation of salt ion in the vacuole is associated with the development of salt-tolerant varieties (Volkmar et al., 1998)

However, storage capacity of roots and the concentration of salt ions in the soil regulate the salt flow and therefore, salt-tolerant traits need fully functional vacuolar accumulation capacity to accumulate salt ions transported from the cytoplasm (Lauchli & Apstien, 1990)

2.2.4. Osmotic adaptation

Osmotic adjustment refers to the increased synthesis of chemical and biochemical molecules in the cytoplasm to establish an osmotic gradient over the vacuolar membrane through the vacuolar compartmentalization of salt ions which is an important mechanism used by plants to reduce salinity stress (Pessarakli, 2014). In this mechanism, plants use conformable solutes viz. proline, glycine-betaine, proline-betaine, B-alaninebetaine, D-sorbitol, D-mannitol, glucose, sucrose, fructose, D-pinitol, L-quebrachitol, Myoinositol, b-dimethylsulfone and propionate (Lauchli& Epstein, 1990). In addition to that, plants also use high concentrations of inorganic ions (Greenway & Munns, 1980). However, although the generation of osmoticum is an action of

plants to overcome salt stress, this mechanism has a negative impact on plant growth due to ion deprivation and toxicity (Munns & Tester, 2008; Volkmar et al., 1998).

2.2.5. Salt exclusion and inclusion

The quantity of salt ions in the stems and roots is usually higher than in the leaves as plants have a selection process for ion absorption (Hale & Orcutt, 1987).

Accumulation of sodium ions can exhibit its toxicity within days or weeks which may cause early death of older leaves. However, the mechanism of roots to keep away sodium is to prevent toxic concentration of sodium ions in the leaves (Munns & Tester, 2008).

Study shows that many salt-tolerant glycophytic species are able to exclude sodium ions from their leaves. Also, crop plants like corn, barley, wheat, bean and chickpea along with some halophytes have the similar mechanism (Volkmar et al., 1998)

One of the important bases to develop salt-tolerant traits is introducing sodium excluding mechanisms in plants since sodium offers toxicity more rapidly than chloride ion. In contrast, chloride ion is referred to as more toxic in some plants like soybean. Basically, plants have some intracellular compartmenting mechanism to tolerate high amounts of sodium and chloride ions (Munns & Tester, 2008).

There is a common phenomenon in which vacuolar volume gets increased in some dicot halophytes due to the accumulation of salt. Also, salt glands or bladders are also produced at the surface of the leaves or stems due to the excretion of sodium and chloride ions. In addition,

several studies showed that, the anatomical adaptation mechanism in some monocot halophytes is only the production of salt glands (Munns & Tester, 2008)

2.2.6. Na+ /K+ inequity

Na+/K+ discrimination is a crucial characteristic to select profit-oriented crop as plant have ion selection potential in which, the take up of Na+ can be replaced by K+ to favour plants to tolerate salinity (Volkmar et al., 1998). In contrast, this approach is not necessarily an important feature in glycophytes. For instance, some salt-tolerant cultivated barley strains and their wild types do not show the enhanced Na+/K+ discrimination trait (Munns & Tester, 2008). However, halophytic plants like to add Na+ as a mechanism to maintain osmotic balance. Moreover, there is an assertive association between Na+ inclusion and salinity tolerance in halophytes (Volkmar et al., 1998).

2.3. Effects of plant probiotic bacteria on improvement of plants tolerance to salinity

The involvement of salt-tolerant genes in plants to increase their tolerance to salinity has been found to be costly and inappropriate to sustainable agriculture. Similarly, pre-treatment of biological materials with some particular chemicals offers the same result. Instead, the use of probiotic bacteria that stimulates plant's growth under stress conditions has been found as a promising strategy to increase agricultural assets in saline areas.

Almost all the rhizospheric components such as rhizospheric bacteria, internal tissue have the capacity to protect plants from various hazards including soilborne pathogens (Sessitsch et al.

2012; Malfanova et al. 2011; Egamberdieva 2008 a, b). Salinity in soil imparts a long-term effect on plants and the eventual consequence is the severe loss in yield (Al-Mutawa, 2003).

However, the inoculation of wheat seeds with *B. amyloliquefaciens*BcA12 and *B. laevolactious* isolated from saline soil showed the increase of root length and shoot length up to 50% in nutrient-deficient saline soil conditions (Egamberdiyeva and Hoflich, 2003). Another study shows that, *B. polymyxa* BcP26 and *B. megaterium* BcM33 have potential to increase the growth of root and shoot of pea plant (18%) and maize (27%), the take up of N and P by 55% as well under dry saline soil condition (Egambardiyeva and Hoflich, 2004). Also, the salt-tolerant *B. pumilus* and *Exiguobacteriumoxidotolerans* induced plant growth and bacoside-A content of brahmi (*Bacopamonnieri*) (Bharti et al., 2013).

Another study shows that rice seeds treated with *B. pumilus* stimulate plant growth under salinity through accelerated activity of some particular antioxidant enzymes and minimizing Na+ deposition in leaves (Khan et al., 2016). Salinity poses negative impacts on *Bassia indica* through various physiological parameters (Hashem et al., 2015a), whereas the inoculation of this species with *B. subtilis* under saline condition can remarkably increase shoot, root growth, total lipid contents along with phospholipid fractions, oleic (Cc18:1), linoleic (C18:2), and linolenic (C18:3) acids and chlorophyll a and b and carotenoid contents on the leaves compared to the control lines. Similarly, radish seeds inoculated with *B. subtilis* and *P. fluorescens* showed a significant increase in fresh and dry mass of leaves and roots, photosynthetic pigments, proline,

the amount of total free amino acids, crude protein, and the uptake of N,P,K+, Ca2+ and Mg2+ compared to the control plants (Mohamed and Gomaa, 2012).

Table 1: Potential of *Bacillus* species in response to salt stress in plants:

Bacterial strain	Plant	References
B. amiloliguefaciens	Wheat	Egamberdiyeva and Höflich (2003)
B. laevolactivus		
B. polymyxa	Pea	Egamberdiyeva and Höflich (2004)
B. megaterium		
B. lentus	Basil	Golpayegani and Tilebeni (2011)
B. pumilus	Rice	Khan et al. (2016)
B. subtilis	Cotton	Egamberdieva and Jabborova (2013)
B.subtilis	Indian bassia	Hashem et al. (2015a)
B. subtilis	Radish	Mohamed and Gomaa (2012)

Table 2: Effects of some other PGPB on salinity tolerance improvement in plants

Bacterial strain	Plant	Effect	Reference
Azospirillum, Pseudomonas syringae, Pseudomonas fluorescens, Enterobacteraerogenes, Rhizobium	Maize	Restricted uptake of Na+ and increased the uptake of K+ and Ca2+. Enhanced nitrate reductase and nitrogenase activity. Increased ACC deaminase activity. Enhanced proline production and decreased electrolyte leakage. Maintained relative water content in leaves and selective take up of K ion	Hamida et al. (2004); Nadeem et al. (2007); Bano and Fatima, (2009)
Pseudomonas fluorescens	Groundnut	Enhanced ACC deaminase activity	Saravanakumar and Samiyappan (2007)
Pseudomonas pseudoalcaligenes	Rice	Increased concentration of glycine betaine.	Jha et al. 2011

Bacterial strain	Plant	Effect	Reference
Pseudomonas putida, *Raoultella planticola* Rs-2	Cotton	Decreased Na+ uptake and increased Mg2+, Ca2+ and K+ ions from soil. Increased ACC deaminase activity	Yao et al. (2010), Wu et al. (2012)
PGPR (Mk1,*Pseudomonas syringae*;Mk20,*Pseudomons fluorescens*; and Mk25,*Pseudomonas fluorescens* biotype G)and *Rhizobium phaseoli* strains M1, M6, and M9	Mung bean	Increased ACC deaminase activity and nodule formation	Ahmad et al. (2011)
Rhizobium phaseoli and PGPR*(Pseudomonas syringae*,Mk1*;Pseudomonas fluorescens*, Mk20 and *Pseudomonas fluorescens* Biotype G,Mk25)	Mung bean	Increased water use efficiency and ACC deaminase activity	Ahmad et al. (2012)

Bacterial strain	Plant	Effect	Reference
Rhizobium and *Pseudomonas*	Mung bean	Increased IAA production and ACC deaminase activity.	Ahmad et al. (2013)
Pseudomonas pseudoalcaligenes and *Bacillus pumilus*	Salt sensitive rice GJ-17	Reduced lipid peroxidation and superoxide dismutase activity	Jha and Subramanium, (2014)
Acinetobacter spp. and *Pseudomonas* sp.	Barley and oats	Production of IAA and ACC deaminase	Chang et al. (2014)

2.4. Mechanism of salinity tolerance enhancement by plant probiotic bacteria

Stress management and the availability of essential nutrients are two basic things which are required for the colonization of microorganisms in the rhizosphere (Matilla et al., 2007). The communities of microorganisms play a vital role in the osmoregulation of halophytes directly or indirectly to cope up with soil salinity. Interestingly, these microorganisms pass a short time or even their whole life-cycle inside the intracellular space of host plants without causing any damage or harm to the host organism (Weyens et al., 2009). However, several recent studies have provided mechanisms that are proved as proficient to maintain the usual maturation of plants in saline conditions. To add, the approaches include the generation and building up of osmolytes to provide a balanced osmotic cellular pressure for the effective cellular metabolism

(Kushwaha et al., 2020). Also, the endophytes dwell in saline soils are proved to be effective to enhance plant's accumulation of osmolytes and antioxidant matters (Vaishnav et al., 2019)

The salt tolerant PGPR (ST-PGPR) has several mechanisms that are directly or indirectly involved in the minimization of salinity stress in plants (Egambardieva et al., 2016; Hashem et al., 2016). Researches ensured that ST-PGPR synthesizes various types of phytohormones (Dodd et al., 2010), produce ACC deaminase (Glick et al., 2007) and also produce exopolysaccharides and osmolytes). In addition, they control plant's defense system by turning on the plant's antioxidative enzymes under salt stress (Hashem et al., 2016)

However, the detail explanation of probiotic mechanisms to enhance salt tolerance in plants is as follows:

2.4.1 Production of 1- Aminocyclopropane-1 Carboxylate (ACC) deaminase

Ethylene is a gaseous growth and stress hormone that is synthesized by almost all plant species (Mayak et al., 2004b; Pierik et al., 2007) and the synthesis of ethylene starts with the production of S-adenosyl-methyonine (SAM). The production of ethylene increases in plants under salinity which is a threat to the plant for its growth. Also, a high level of ethylene in nodules imposes adverse impact on N_2 fixation (Ma et al., 2002). Therefore, to minimize the harmful effects of ethylene, plants are usually treated with probiotic bacteria that produce ACC deaminase (Glick, 2005; Etesami et al., 2015). The synthesis of ACC deaminase enzyme is the most important mechanism for the direct enhancement of plant growth by PGPRs. Bacterial Indole-3-acetic acid (IAA) decrease the formation of ethylene in plants by accelerating the activity of ACC deaminase, catalyzing the hydrolysis of ACC (an ethylene precursor produced by plants) to ammonia and α-ketobutyric acid (Etesami and Beattie, 2017).

In addition, there is a correlation between the synthesis of ACC deaminase and Indole-3-acetic acid (IAA). The combination of plant produced IAA along with PGPR produced IAA enhances plant growth and activates the transcription of the enzyme ACC synthase. Therefore, there is an increased level of ethylene that feedback inhibits IAA signal transduction and thus limits IAA-catalyzed plant growth. In addition, ACC deaminase containing PGPR decreases the feedback inhibition by lowering the ethylene level. Here, IAA signal transduction carries on with the development of the plant while avoiding the accumulation of large amounts of ethylene (Ma et al., 2020).

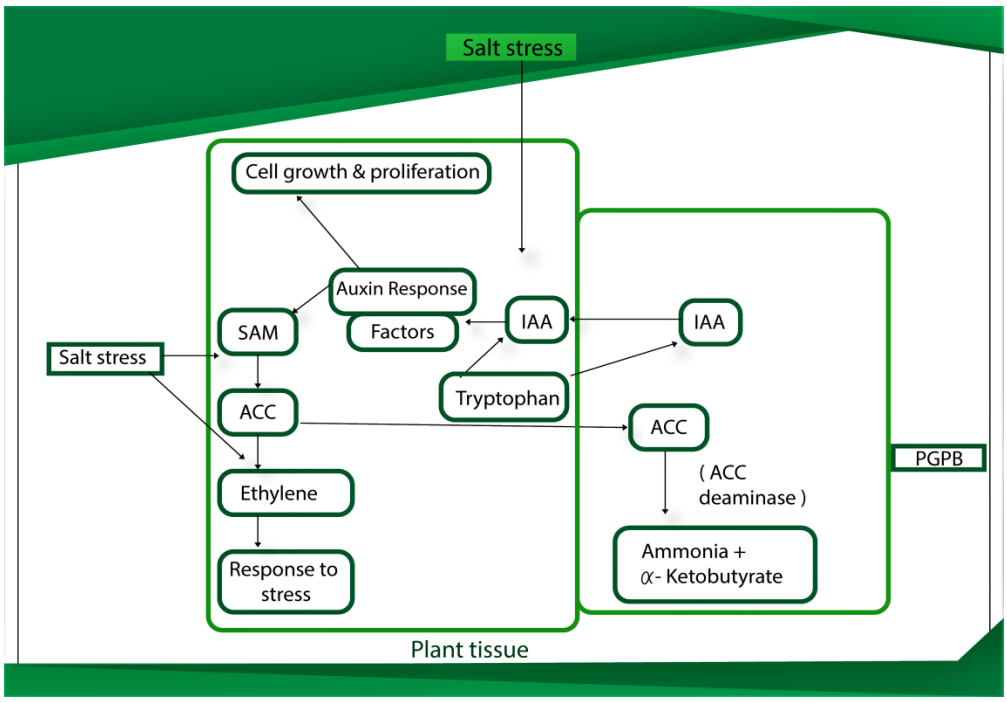


Figure2: PGPRs mechanism in lowering plants ethylene level through producing ACC deaminase and IAA

Inoculation of plants with ACC deaminase producing bacteria result in reduced ethylene formation, higher root growth and enhanced resistance to salinity (Cheng et al., 2007; Etesami and Beattie, 2017; Mayak et al., 2004a,b; Glick et al., 2007; Nadeem et al., 2010; Barnawal et al., 2012; Jha et al., 2012; Zahir et al., 2009). In addition, these PGPRs also influence the homeostasis of plant ethylene by changing the expression of genes that encode the ethylene synthesis enzyme ACC synthase and ACC oxidase (Tsukanova et al., 2017). A number of scientific research have emphasized on the performance of PGPR with ACC deaminase to minimize the harmful effects of increased ethylene level caused by salt stress. In particular, 25 of 140 halotolerant bacterial strains isolated from the soil of coastal areas of South Korean Yellow Sea offer high levels of ACC deaminase function (Siddikee et al., 2010). Moreover, PGPR that producesACC deaminase accelerates essential nutrient uptake such as N, P and K that results in the increase of K^+/Na^+ ratios in salt stressed plants (Nadeem et al., 2009).

2.4.2 Production of phytohormones

Indole-3-acetic acid (IAA) synthesis is directly related with the growth of plants. Also, it falls in the auxin class and it is the most usual plant hormone. An effector molecule IAA works in between bacterial-bacterial interaction and bacteria and plants that produce IAA (Spaepen and Vanderleyden, 2011). Along with seed germination and root system development, IAA increases plants tolerance to salinity stress (Aeron et al., 2011). As a result, plants get a large root surface area that ensures higher intake of nutrients from soil (Boiero et al., 2007). Several researches have shown the positive impact of IAA produced by the PGPRs on plants under saline conditions. For instance, salt tolerant *B. subtilis* maintains the regular growth of Indian bassia by

providing adequate amounts of IAA and it also reduces the ethylene level under saline condition (Otlewska et al., 2020). Similarly, Abeer et al. (2015) shows that, this bacterial strain increases the phospholipid fraction, total lipid content, photosynthetic pigments, oleic, linoleic and linolenic acids in plant leaves while inoculated in plants under saline condition.

Besides these, auxin activates the transcription of multiple genes that are known as primary auxin response genes in Arabidopsis, soybeans and rice (Hagen and Guilfoyle, 2002). Also, this hormone has an adverse impact on the control of the expression of the rice gene adenosine phosphate isopentenyltransferase (OsIPT) which encodes a major enzyme in CTK biosynthesis in nodes and this way it inhibits the growth of tiller buds in rice (Liu et al., 2011).

Cytokinins (CKs) have important functions in abiotic and biotic stress management by plants (Dodd et al., 2010). Production of cytokinins is an usual phenomenon by PGPRs to resist salt entrance in plants. The CK synthesizing capacity by PGPRs or the ability of changing plant CK homeostasis focuses the importance of how PGPRs protect plants from salinity stress.

Gibberellic acid (GA) maintains several vital physiological characteristics in plants and GA signaling is a crucial factor in the inhibition of plant growth under hazardous conditions (Magome and Kamiya, 2016; Martínez et al., 2016). Several specific PGPRs are able to influence the production of GA in plants, thus the treatment of plants with these PGPRs increase the level of GA synthesis in shoots under salt stress (Kang et al., 2014a).

Abscisic acid (ABA) is usually produced in response to abiotic stresses which is an important plant stress hormone. Also, this hormone activates the expression of genes that are responsible for stress resistance (Sah et al., 2016). Along with performing an important function in plant-PGPR association (Dodd, 2003), this hormone facilitates plants capacity to reduce salt stress by mediating stomatal and thereby photosynthetic responses to severe salt stress (Dodd and Pérez - Alfocea, 2012). Usually, ABA producing PGPRs adjust the level of ABA status in plants to offer a strong response in terms of salt stress. Moreover, there are large numbers of PGPRs that synthesize ABA in vitro (Dodd et al., 2010). To add, these PGPRs increase plant growth under saline conditions by producing ABA (Naz et al., 2009).

However, several researches showed that PGPRs can produce phytohormones that help plants to protect themselves from salinity stress, but how the PGPRs actually perform those mechanisms are still not fully discovered. Therefore, further study is required to reveal how PGPRs influence the process.

2.4.3 Biological Nitrogen (N_2) fixation

Ensuring the availability of biological nitrogen by plants (especially legumes) is very important for productivity. In saline soils, salinity competes with nitrogen and thus makes nitrogen less available to the plant (Naidoo, 1987). To overcome this problem, utilization of PGPRs can be very useful as they can fix nitrogen by symbiotic and non-symbiotic mechanisms (Saghafi et al., 2019).

In symbiotic mechanisms, the symbiotic nitrogen-fixing bacteria occupy the root hairs of the host plant, getting higher in numbers and influence the root nodule formation and stimulate the

interaction between plant cell and bacteria. Also, the bacteria transform free nitrogen to ammonia within the nodule and the host plant uses them for its growth and development. However, to maintain optimum growth and nodule formation of legumes, seeds are often inoculated with PGPRs, especially in soils lacking the required bacterium (Encyclopedia Britannica).

In addition, the symbiotic approach increases the level of nitrogen content up to 65% of the total nitrogen assimilation by plants (Razwar et al., 2013). Usually, farmers use chemical fertilizers that eventually result in the increase of salinity, loss of land fertility and changes in the functions of soil-microflora (Akhavan-Kharazian et al., 1991; Rueda-Puente et al., 2003). On the other hand, use of PGPRs having nitrogen-fixing capacity can be a useful alternative to the chemical fertilizers especially in saline areas as they impose higher osmotic tolerance through producing osmolytes which allow them to control their cell metabolism and turgor (Yan et al., 2015)

Besides these, PGPRs that are salt-tolerant and have N_2 fixing capacity are key sources of free nitrogen in saline soils. Moreover, the amount of nitrogen fixed by these bacteria has been estimated as 20-30 kg h^{-1} $year^{-1}$ (Oberson et al., 2013)

2.4.4 Phosphate solubilizing by the Probiotics

Phosphorus (P) is an essential macronutrient for plants and although both organic and inorganic phosphorus is available in adequate amounts in soils, its access to the plants is limited because of its insoluble form. To add, only 0.1% (w/w) of the total P is supplied to the plants because of its poor fixation and solubility whereas, this compound makes up about 0.05% (w/w) of soils (Goldstein, 1986). Besides these, irrigation with saline water and use of inorganic fertilizers reduce soil nutrient and increase salt concentration. Moreover, salinity leads to depletion and

sedimentation of absorbable phosphorus. On the contrary, PGPRs with phosphate solubilizing capacity offer an opportunity to increase phosphate availability in plants without aggravating the salinity level (Etesami and Beattie 2018). These special probiotics use various mechanisms such as ion exchange, chelation and acidification by excreting low molecular weight organic acids (Sharma et al., 2013). For example, Myak et al., (2004a) and Upadhyay and Singh, (2015) reported that phosphate-solubilizing PGPRs solubilize insoluble P in saline soils. Also, 129 bacterial strains have been identified that are able to solubilize rock phosphate through the screening of the mangrove *A. marina* (El-Tarabily and Youssef, 2010).

2.4.5 Production of Siderophore

Iron is also an essential micronutrient which is an important constituent of many enzymes that catalyze several vital biochemical processes including photosynthesis, respiration and N_2 fixation (Kobayashi and Nishizawa, 2012; Abbas et al., 2015). All over the world, the soil rich with calcium carbonate ($CaCO_3$) and sodium (Na) have very poor amounts of iron (Rabhi et al., 2007; Abbas et al., 2015). PGPRs and plant growth promoting fungus (PGPFs) can be utilized as they are able to produce siderophores which are small and chelate iron with higher affinity (Abbas et al., 2015; Etesami and Beattie, 2018). Moreover, these siderophores bind with iron, make iron-siderophore complex and become available for plants in need (Kloepper et al., 1980).

However, bacterial siderophores have greater attraction for iron compared to the fungal pathogens that need iron for several important cellular functions and plant's infecting strategies (Miethke and Marahiel, 2007). Besides these, Labuschagne et al. (2010) reported the potential

use of bacterial strains that produce siderophores that are effective for the suppression of fungal pathogens of rice and wheat.

2.4.6 Antioxidant activity

When the salt concentration in soil is too high, plants produce reactive oxygen species (ROS) such as, hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH) and alkaline radicals (Otlewska et al., 2020). This phenomenon poses negative impacts on crucial biomolecules such as DNA, protein, lipids etc. whereas the eventual consequence is plant cell damage and early senescence or even necrosis (Møller et al., 2007; Miller et al., 2010; Habib et al., 2016; Zhang et al., 2018). Although the ROS is produced by plants at a moderate level under optimal growth conditions, the synthesis gets higher under stress conditions (Miller et al., 2010). Hossain and Dietz (2016) and Hossain et al., (2017) proved that the plasma-membrane bound NADPH oxidase that regulates cellular redox homeostasis under salt stress is the main system that is responsible for ROS production.

There are many plant cell components which are associated in maintaining the intracellular ROS levels. From the components, antioxidant enzymes viz. superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDAR), glutathione peroxidase (GPX) or glutathione s-transferase (GST) are the key role player (Yan et al., 2013; Hossain and Dietz, 2016; Sukweenadhi et al., 2018).

The consequences of oxidative stress can be minimized by the treatment of plants with antioxidative enzymes producing ST-PGPR (Manaf and Zayed 2015; Islam et al., 2016). Also,

Jha and Subramanian (2013); Sen and Chandrasekhar (2015); Ansari et al. (2019) reported that plant inoculation with ST-PGPRs results in higher production of antioxidant enzymes. For example, the increase of catalase activity in tissue of lettuce along with the decrease of oxidative damage has been observed (Kohler et al., 2009). Moreover, Patel and Saraf (2013) showed the positive impacts in Jatropha leaves through the inoculation of some ST-PGPRs such as *Pseudomonas pseudoalcaligenes, Enterobacter cloacae* and *Bacillus sp*. under salt stress. Besides these, in Tunisian saline soils, inoculation with *Pseudomonas sp*. and *Bacillus sp*. strains in salt tolerant plant *Sulla carnosa* offered improved growth and stress tolerance. Here, the bacterial strains reduced the plant's stomatal conductance and regulated antioxidant activities so that the plant can achieve optimal growth and nutrition under salt stress. (Hidri et al., 2016)

Assessment of the current literature reveals the potential of halotolerant plant growth promoting bacteria that exhibits essential functions such as ACC deaminase production, synthesis of phytohormones, siderophore production, production of antioxidants, fixation of biological nitrogen in legumes etc. Practicing agriculture with the association of these PGPRs is a much more effective approach in saline areas. In Bangladesh, there is very little research regarding the enhancement of salinity tolerance in rice by probiotics. Therefore, conducting this research in a large area can ensure a sustainable agricultural system in the coastal areas over the country.

CHAPTER III

MATERIALS & METHODS

3.1 Collection of plant samples

Plant samples were collected from four coastal districts of Bangladesh (Chattogram, Cox's Bazar, Lakshmipur and Noakhali). Sample collection areas were selected on the basis of salinity in soil. The variety of plant samples collected from Chattogram and Noakhali was BRRI dhan 29. Also, Cox's bazar and Lakshmipur plant samples were BRRI dhan 28. All plant samples were salt-susceptible and the plant varieties were confirmed by corresponding field farmers. Seeds were collected from Bangladesh Rice Research Institute (BRRI), regional station, Cumilla and Bangladesh Institute of Nuclear Agriculture (BINA), substation-Cumilla. The seed samples were selected on the basis of salinity tolerance. Two rice varieties were selected, one is salinity susceptible, that is BRRI dhan 29 and the other one is salinity tolerant which is BINA dhan 10.

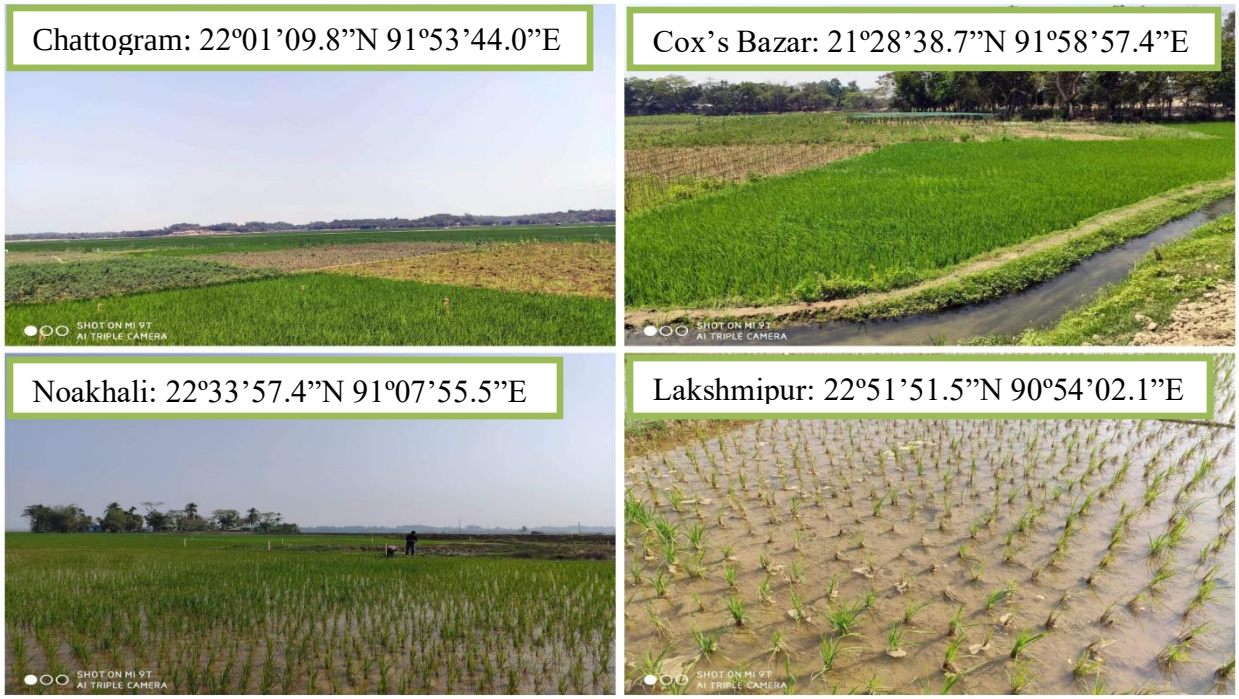


Figure 3: Plant sample collection area (along with district’s GPS coordinates) of Bangladesh

3.2 Place of study

The research was accomplished at the Institute of Biotechnology and Genetic Engineering (IBGE), Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur.

3.3 Duration of study

The study started from March 2021 and ended in July 2021.

3.4 Isolation of bacteria

For the collection of potential salt tolerant bacteria, different plant parts such as leaf, root and rhizospheric soil were taken. However, after washing, the plant parts were surface sterilized with 70% ethanol for 3 minutes and then 1% sodium hypochlorite for 1 minute. The plant parts were further sterilized with 100% ethanol for 5 minutes and then crushed with the help of mortar & pestle to obtain endophytic bacteria. Six-fold serial dilution was prepared in autoclaved water. Of them; fifty microliter aliquots from particular dilution were taken through pipette and then spread on nutrient broth agar (NBA) plates and then placed in the incubator for 24 hours at 26° C±1ºC. After incubation, different bacterial colonies were isolated and purified on NBA medium.

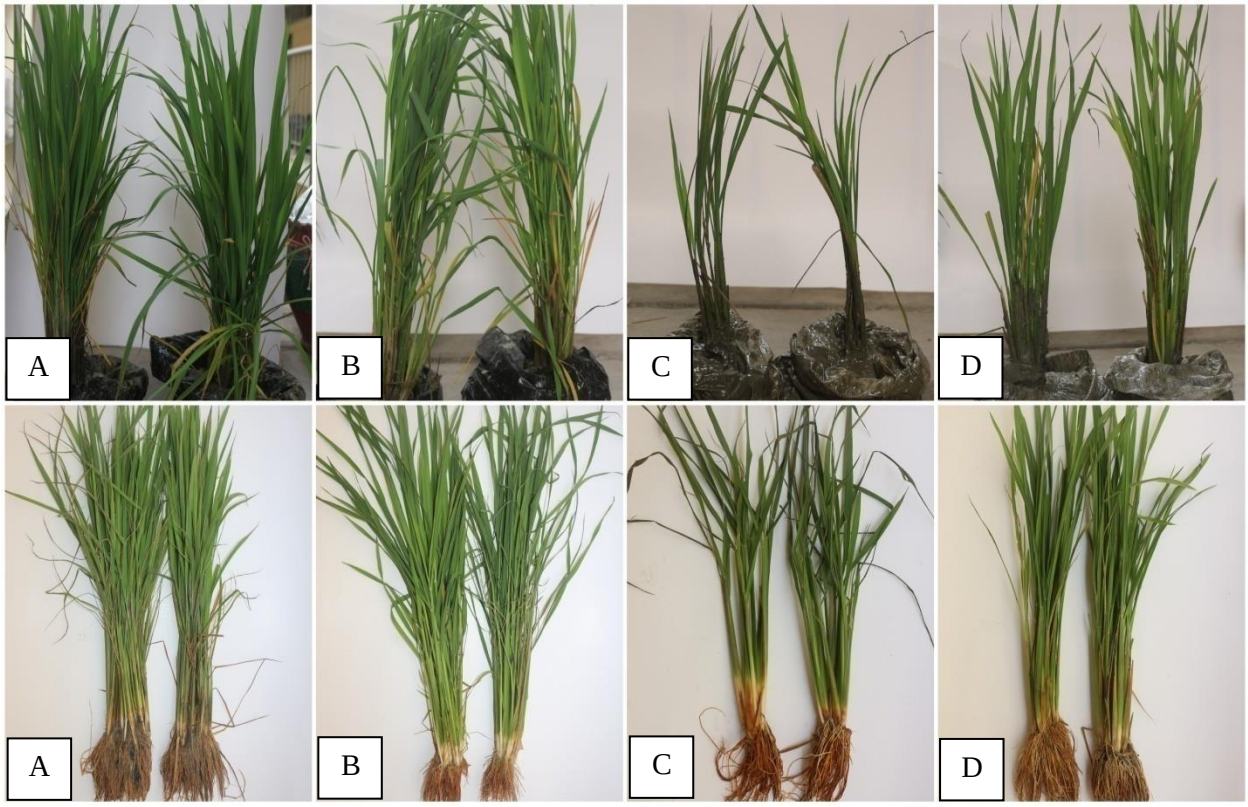


Figure 4: Collected plant samples for probiotic isolation (A) Chattogram, (B) Cox's Bazar, (C) Lakshmipur and (D) Noakhali

3.5 Screening of bacteria

For salt tolerance evaluation, the isolated bacteria were grown in NBA media with various doses of Sodium Chloride (NaCl) (2%, 4%, 6%, 8%, 10% and 12%). Then the development of bacteria in media with different concentrations of salt was observed.

3.6 NaOH test

A NaOH test was done to identify whether the bacteria were gram positive or gram negative. To conduct this test, a loop full of bacteria was taken on to a glass slide and mixed with a drop of 3% NaOH solution to make a smear. After a few moments, those isolates produced sticky characteristics were gram negative and the non-sticky were gram positive.

3.7 Plant growth enhancing capacity of bacterial isolates

3.7.1 Preparation of bacterial inocula

Bacterial isolates were cultured in 250 ml conical flasks containing 200ml NB broth medium on a shaker at 120 rpm for 72 hours at 27ºC. To collect bacterial cells, the broth was centrifuged at 14000 rpm for 1 minute at 4ºC and two times washed with SDW. The bacterial pellets were suspended in around 1 ml SDW and vortex for 45 seconds before using for the seed treatment.

3.7.2 Seed treatment with bacteria

One gram of surface sterilized seeds was soaked into bacterial suspension. The bacteria treated seeds were dried overnight at room temperature to ensure better coating of the seeds with bacteria.

3.7.3 Seed germination in Petri dish

The inoculated seeds were placed on a Petri dish containing water-soaked sterilized filter paper. After the germination of seeds, the seedlings were allowed to grow for one week. The seedlings were watered on alternate days.

3.8 Statistical analysis

The statistical method includes frequency tables and graphs, whereas the graphs were prepared by MS Excel.

3.9 Polymerase chain reaction (PCR) and Genomic DNA extraction

For the extraction of DNA, endophytes were first cultured in nutrient broth for 24 hours. 1 ml of overnight culture was taken into a 1.5 ml microcentrifuge tube followed by centrifuging at 16000 rpm for 2 minutes to pellet the cells and the supernatant was removed. Then the cells were resuspended thoroughly in 480 µl of 50 mM EDTA. Then 10mg/ml lysozyme was added to resuspend the cell pellet and mixed gently. Then the samples were incubated at 37ºC for 60 minutes followed by centrifuging for 2 minutes at 16000 rpm and the supernatant was removed. 600 µl of nuclei lysis solution was added and mixed gently. The samples were then incubated at 80ºC for 5 minutes for efficient cell lysis and then cooled to room temperature. 3 µl of RNAse solution was added and inverted 2/3 times for mixing. Then the samples were again incubated at 37ºC for 60 minutes followed by cooling to the room temperature. 200µl of protein precipitation solution was added to RNAse-treated cell lysate and vigorously vortexed for mixing. Then the samples were incubated on ice for 5 minutes followed by centrifuging at 16000 rpm for 3 minutes. After that, the DNA containing supernatant was transferred to a new 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. Then the tubes were gently mixed by inversion until thread-like strands of DNA formed a visible mass. Then the samples were centrifuged at 1600 rpm for 2 minutes. After that, the supernatant was drained and cleaned carefully on clear absorbent paper. Then 600 µl of 70% ethanol was added to wash the

DNA through inversion of tubes. After that, the samples were again centrifuged at 16000 rpm for 2 minutes and the ethanol was removed carefully. The pellets were allowed to air dry for 15 minutes and then 20 µl of DNA rehydration solution was added. Finally the DNA samples were kept in -20ºC in a low temperature freezer for further use.

The 16S rRNA gene of the bacterial isolates was amplified using two universal primers 27F and 1492R.

Table 3: Primer used for molecular identification of bacterial isolates

Target gene	**Primer**	**Primer sequence (5´-3´)**	**Length (bp)**
16S rRNA	27F 1492R	AGAGTTTGATCCTGGCTCAG GGATACCTTGTTACGACTT	20 19

Individual PCR reaction mixture contained nuclease free water, buffer, dNTPs, forward primer, reverse primer, Taq polymerase and sample DNA. The compositions of the PCR reaction mixer are given in the following table. PCR amplification was performed in the Veriti® 96-Well thermal cycler (Applied Biosystems, Foster City, California, United States)

Table 4: Composition of PCR reaction mixer

Component	Amount
Nuclease free water	76 µl
Buffer	12 µl
dNTPs	4 µl
Forward Primer	2 µl
Reverse Primer	2 µl
Taq polymerase	2 µl
Template DNA	2 µl

For PCR amplification, the initial denaturation, annealing and extension temperature was 95ºC, 56ºC AND 72ºC respectively. The temperature profile used in this study is as follows:

Table 5: Conditions for PCR amplification of the target gene:

Target gene	Initial denaturation	35 cycles			Final extension
		Denaturation	Annealing	Extension	
16S rRNA	95ºC, 2 min	95ºC, 30s	56ºC, 30s	72ºC, 1.25 min	72ºC, 5 min

CHAPTER IV

RESULTS

Two experiments were conducted (i) collection of rice plant samples from saline soils in different districts and isolation and purification of the salinity tolerant bacteria from rice; and (ii) evaluation of the performances of some saline tolerant isolates on seedling growth of rice cv. BRRI dhan 29 (a salinity susceptible variety) and BINA dhan 10 (a salinity tolerant variety). The results of these experiments are described in this chapter.

4.1. Screening of salinity tolerant bacteria

To identify the effective saline tolerant bacterial strain, isolated bacterial strains were cultured in different salt concentrations. The concentration of NaCl was 2%, 4%, 6%, 8%, 10% and 12% (w/v). The growth of the bacteria was recorded. 41 bacterial isolates were cultured; of them BTNSo1 –BTNSo8 (8 bacteria) were from saline soil of Noakhali, BTCoR1-BTCoR4, BTCoL1-BTCoL1-BTCoL6, BTCoSo1-BTCoSo6 (16 bacteria) were from Cox's bazar, BTChL1, BTChL2, BTChR1, BTChR2, BTChSo1, BTChSo2, (6 bacteria) were from Chattogram and the rest BTLSo1-BTLSo10 and IBGE3C (11 bacteria) were from Lakshmipur district. The initials; Co, Ch, L and N represents Cox's Bazar, Chattogram, Lakshmipur and Noakhali respectively. Whereas, L, R and So represents leaf, root and soil accordingly. Among 41 bacterial isolates, only 3 bacterial isolates provided better growth in 12% NaCl. Current study involved three replications of each experiment. However growth of the bacteria in nutrient broth agar (NBA) medium with different conc. of NaCl (w/v) is presented in the following table.

Table 6: Growth of isolated bacterial strain in NBA media with different salt concentration

Name of isolates	% NaCl Concentration (w/v)						
	Control	**2%**	**4%**	**6%**	**8%**	**10%**	**12%**
BTNSo1	+++	+++	+++	++	NG	NG	NG
BTNSo2	+++	++	+++	+++	NG	NG	NG
BTNSo3	+++	+++	+++	+++	NG	NG	NG
BTNSo4	+++	+	+	++	NG	NG	NG
BTNSo5	+++	+++	+++	+++	++	++	+
BTNSo6	+++	+++	+++	+++	NG	NG	NG
BTNSo7	+++	+++	+++	+++	NG	NG	NG
BTNSo8	+++	++	++	+	NG	NG	NG
BTCoR1	+++	+++	+	+	NG	NG	NG
BTCoR2	+++	+++	+++	++	+++	++	++
BTCoR3	+++	++	+++	+++	+++	++	+
BTCoR4	+++	+++	+++	+++	+	NG	NG
BTCoL1	+++	+++	+++	+++	+++	+	NG
BTCoL2	+++	+++	+++	+++	+++	+	NG
BTCoL3	+++	+++	+++	++	++	+	NG
BTCoL4	+++	+++	++	+	NG	NG	NG
BTCoL5	+++	+++	++	++	+++	+	NG
BTCoL6	+++	+++	+++	+	++	+	NG
BTCoSo1	+++	+++	+++	+	+	NG	NG

Name of isolates	% NaCl Concentration (w/v)						
	Control	2%	4%	6%	8%	10%	12%
BTCoSo2	+++	+++	+++	+++	+++	+++	++
BTCoSo3	+++	+++	+++	+++	NG	NG	NG
BTCoSo4	+++	+++	+++	+	+	NG	NG
BTCoSo5	+++	+++	+++	+	NG	NG	NG
BTCoSo6	+++	+++	+++	+	NG	NG	NG
BTChL1	+++	+++	+++	+++	+++	+	NG
BTChL2	+++	+++	+++	+++	+++	+	NG
BTChR1	+++	+++	++	+	+	NG	NG
BTChR2	+++	+++	+++	++	++	+	NG
BTChSo1	+++	+++	+++	+++	NG	NG	NG
BTChSo2	+++	+++	+++	++	NG	NG	NG
BTLSo1	+++	+++	++	+	NG	NG	NG
BTLSo2	+++	+++	+++	+	NG	NG	NG
BTLSo3	+++	+++	+++	++	NG	NG	NG
BTLSo4	+++	+++	+++	+++	NG	NG	NG
BTLSo5	+++	+++	+++	++	NG	NG	NG
BTLSo6	+++	+++	+++	++	NG	NG	NG
BTLSo7	+++	+++	+++	++	+	NG	NG
BTLSo8	+++	++	+++	+++	+	NG	NG
BTLSo9	+++	+++	+++	++	+	NG	NG
BTLSo10	+++	+++	+++	+++	++	++	+

Name of isolates	% NaCl Concentration (w/v)						
	Control	**2%**	**4%**	**6%**	**8%**	**10%**	**12%**
IBGE3C	+++	++	+++	+++	+++	+++	++

Note: "+++" represents high growth, "++" moderate growth, "+"low growth and "NG" no growth.

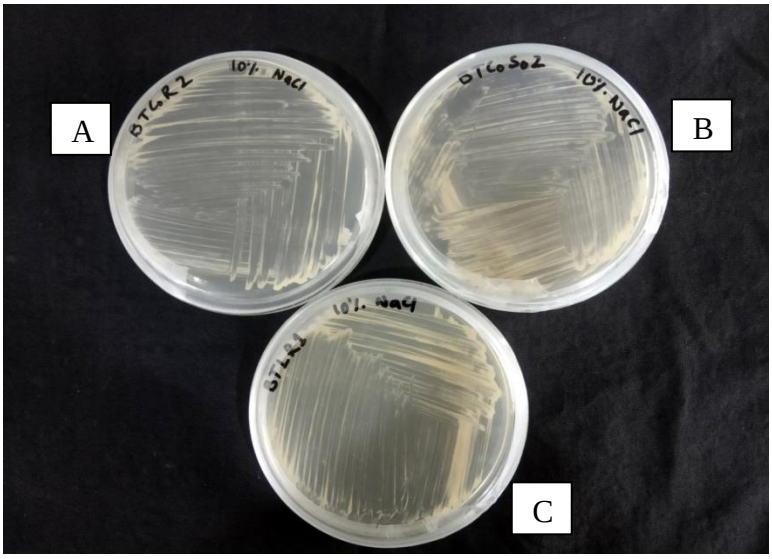


Figure 5: Growth of 3 better grown isolates (A) BTCoR2, (B) BTCoSo2 and (C) IBGE3C in nutrient broth agar (NBA) medium with 10% NaCl (w/v)

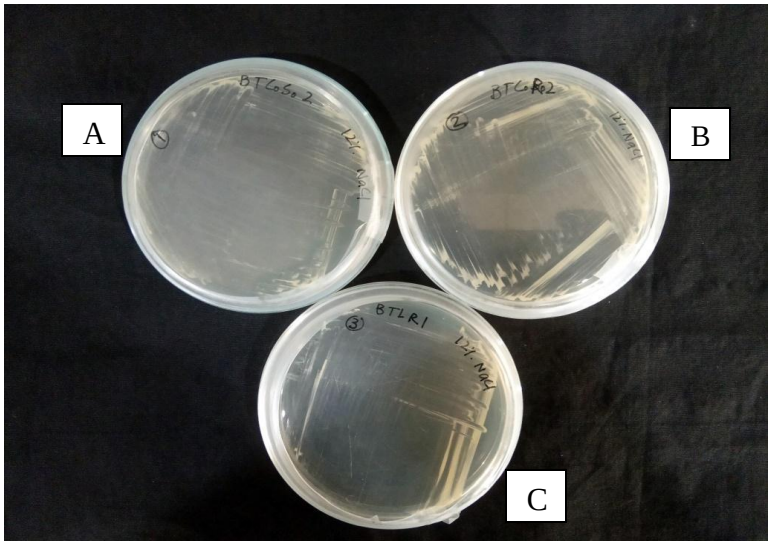


Figure 6: Growth of 3 better grown isolates (A) BTCoR2, (B) BTCoSo2 and (C) IBGE3C in nutrient broth agar (NBA) medium with 12% NaCl (w/v)

4.2. NaOH test

Among 3 isolates, BTCoSo2 was gram negative and the rest two (BTCoR2 and IBGE3C) were gram positive.

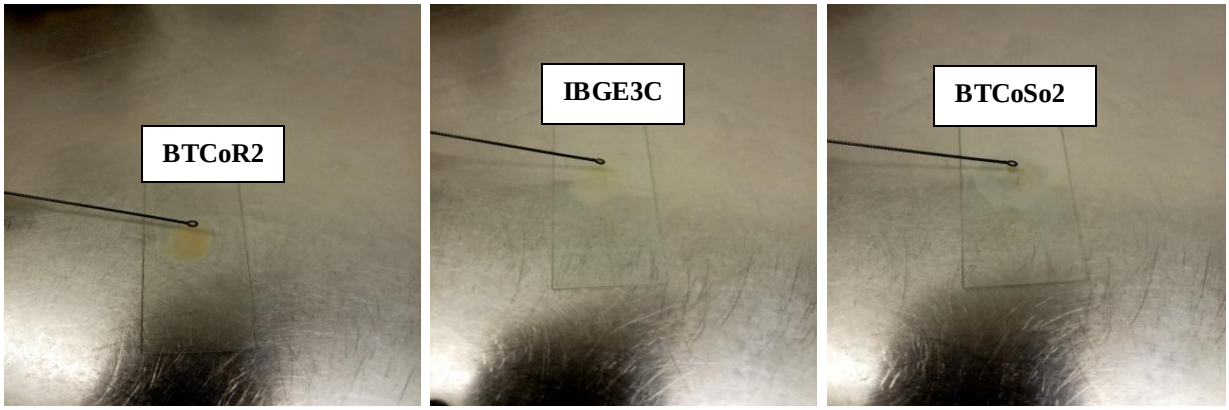


Figure 7: NaOH test result of 3 bacterial isolates

4.3. Colony characteristics of PGPB

Colony characteristics along with growth patterns of the isolates were recorded. All 3 well performers produced mat-like structure and of them; BTCoSo2 was white and BTCoR2 & IBGE3C was light brownish in color. These results suggest that the bacteria collected from saline soil offered diverse color in NBA medium.

Table 7: Colonial characteristics of the bacterial isolates

Bacterial isolates	**Morphological character**	
	Colony color	**Margin**
BTCoSo2	White	Irregular
BTCoR2	Light brown	Irregular
IBGE3C	Light brown	Irregular

4.4 Effect of bacterial isolates on Seedlings in Petri dish (BRRI dhan 29)

There was a significant variation in the effect of root and shoot lengths through the effects of bacteria at 7 days after inoculation. Highest root length was observed in IBGE3C at 0.2% salinity and lowest root length was observed in control at 1% salinity. Similarly, highest shoot length was observed in BTCoR2 at 0.4% salinity and lowest shoot length was seen in control at 1% salinity.

In addition, IBGE3C produced the highest root dry weight at 0.2% salinity and lowest root dry weight was found in control at 1% salinity. Also, highest shoot dry weight was produced by IBGE3C at 0.2% salinity and lowest shoot dry weight was recorded in control at 1% salinity.

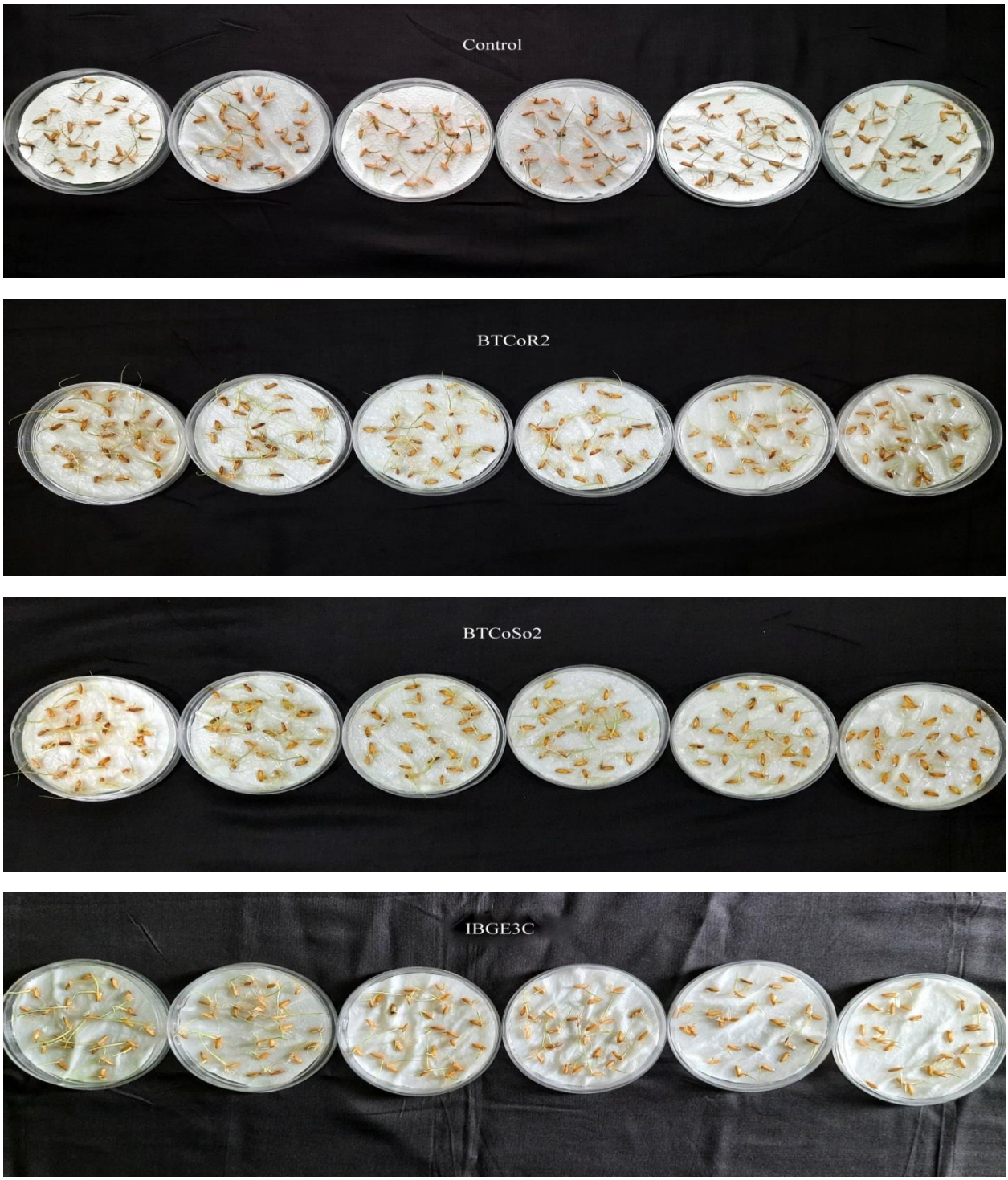
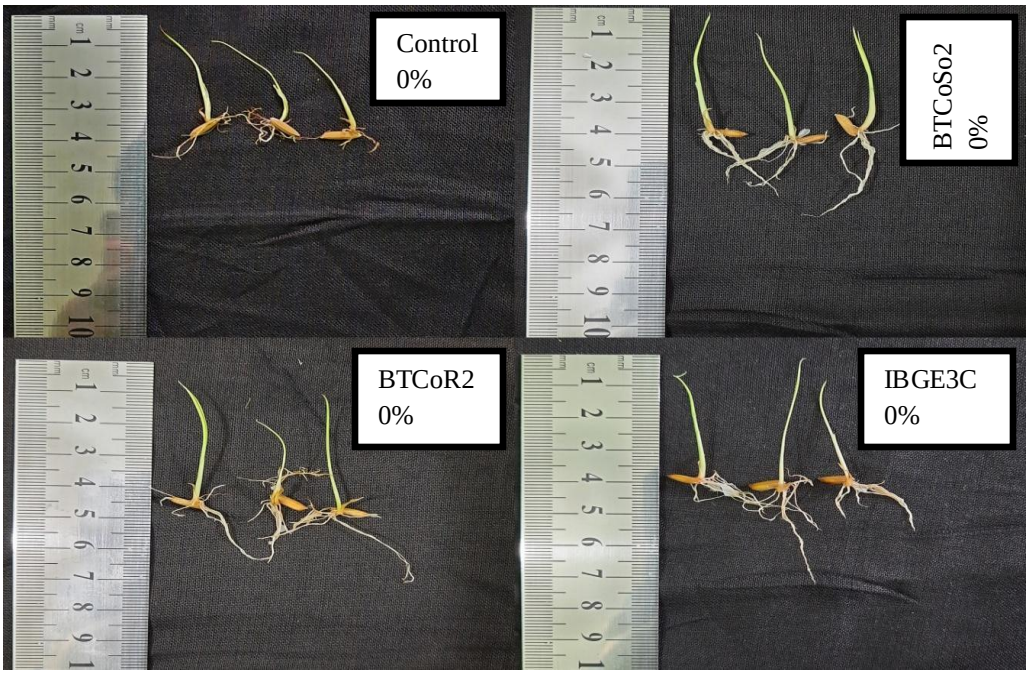


Figure 8: Assessment of BRRI dhan 29 rice seedling in Petri dish (from left to right: 0%, 0.2%, 0.4%, 0.6%, 0.8% and 1% NaCl (w/v)



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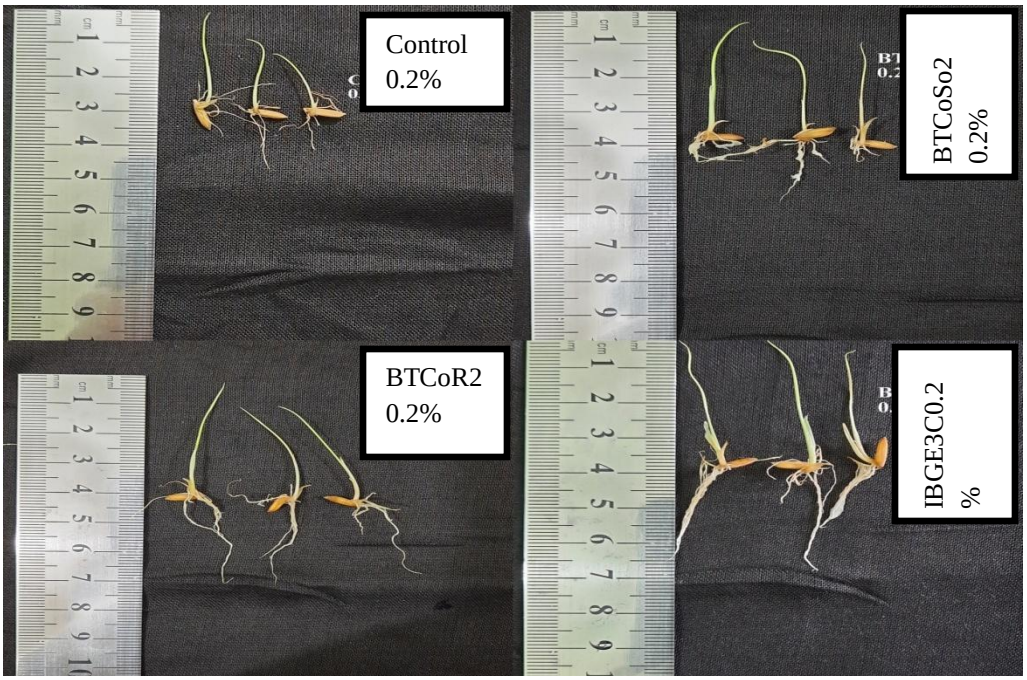

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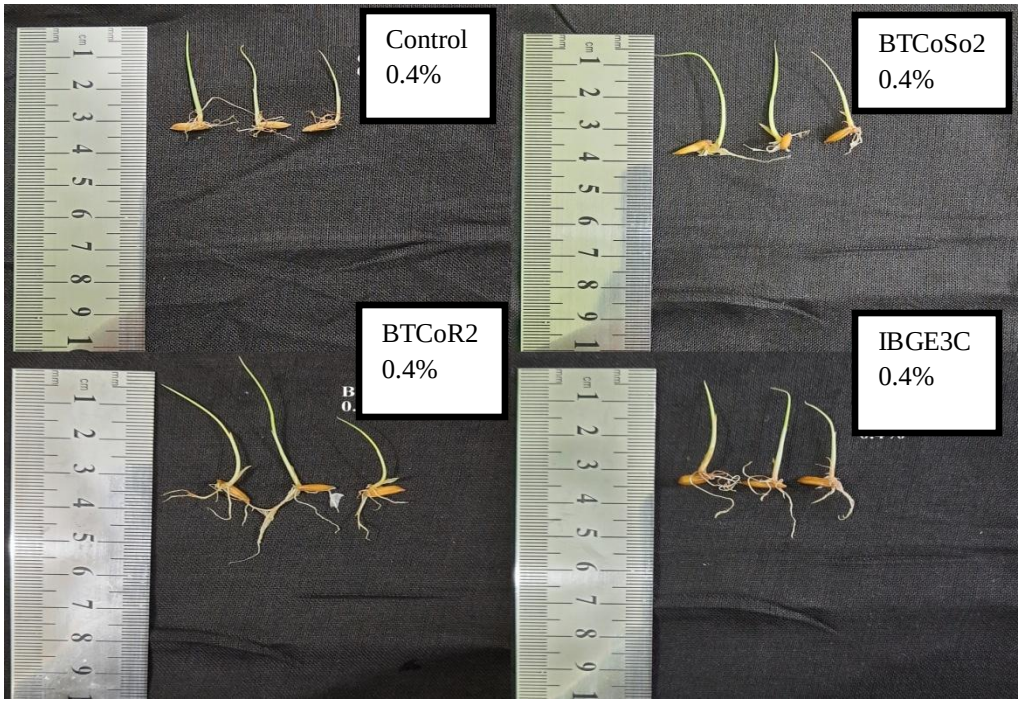

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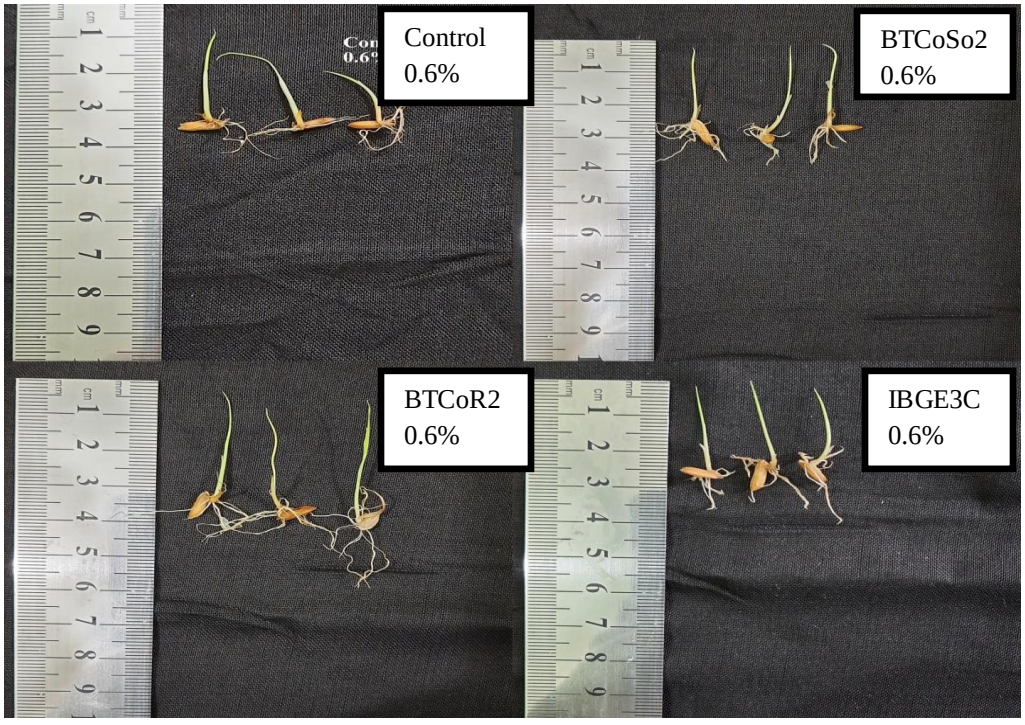

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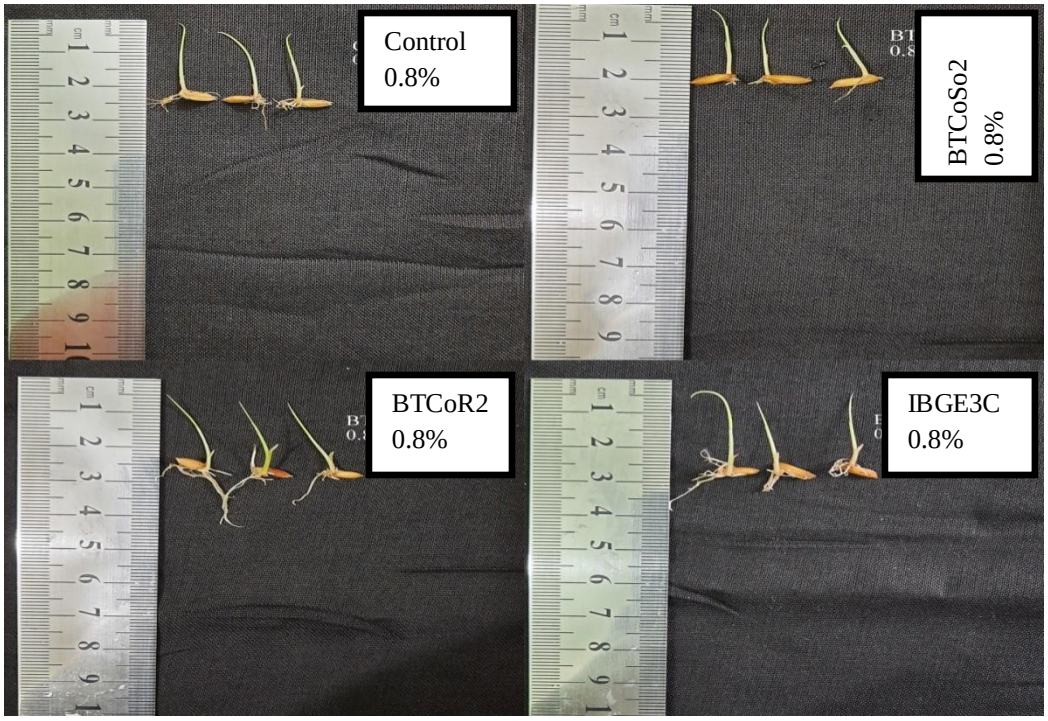

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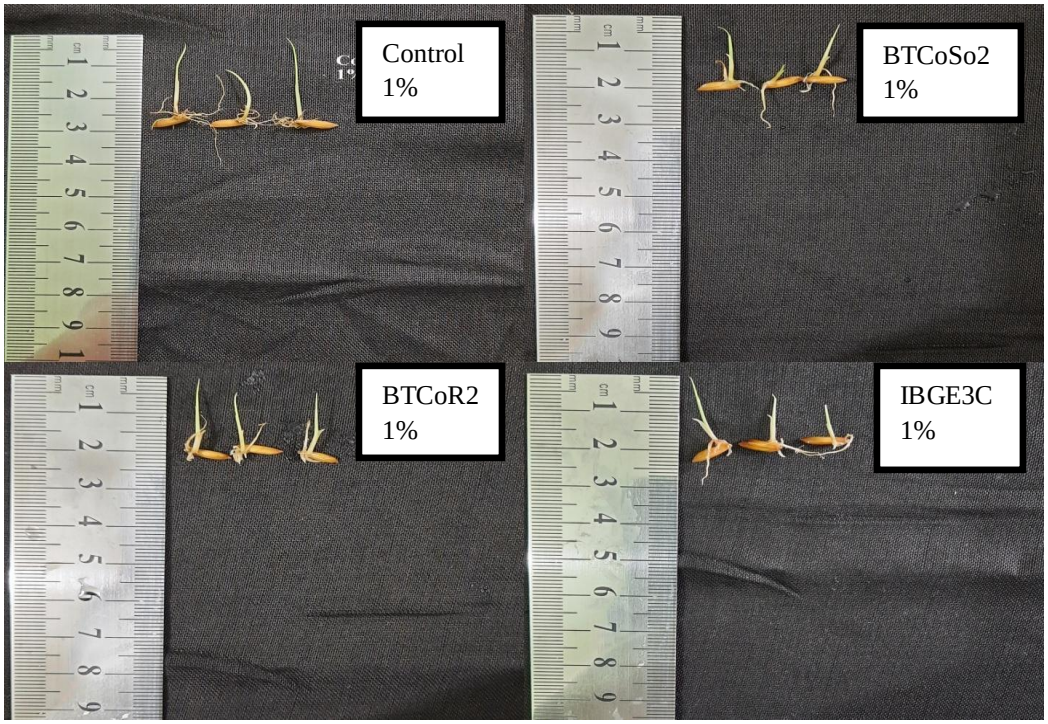

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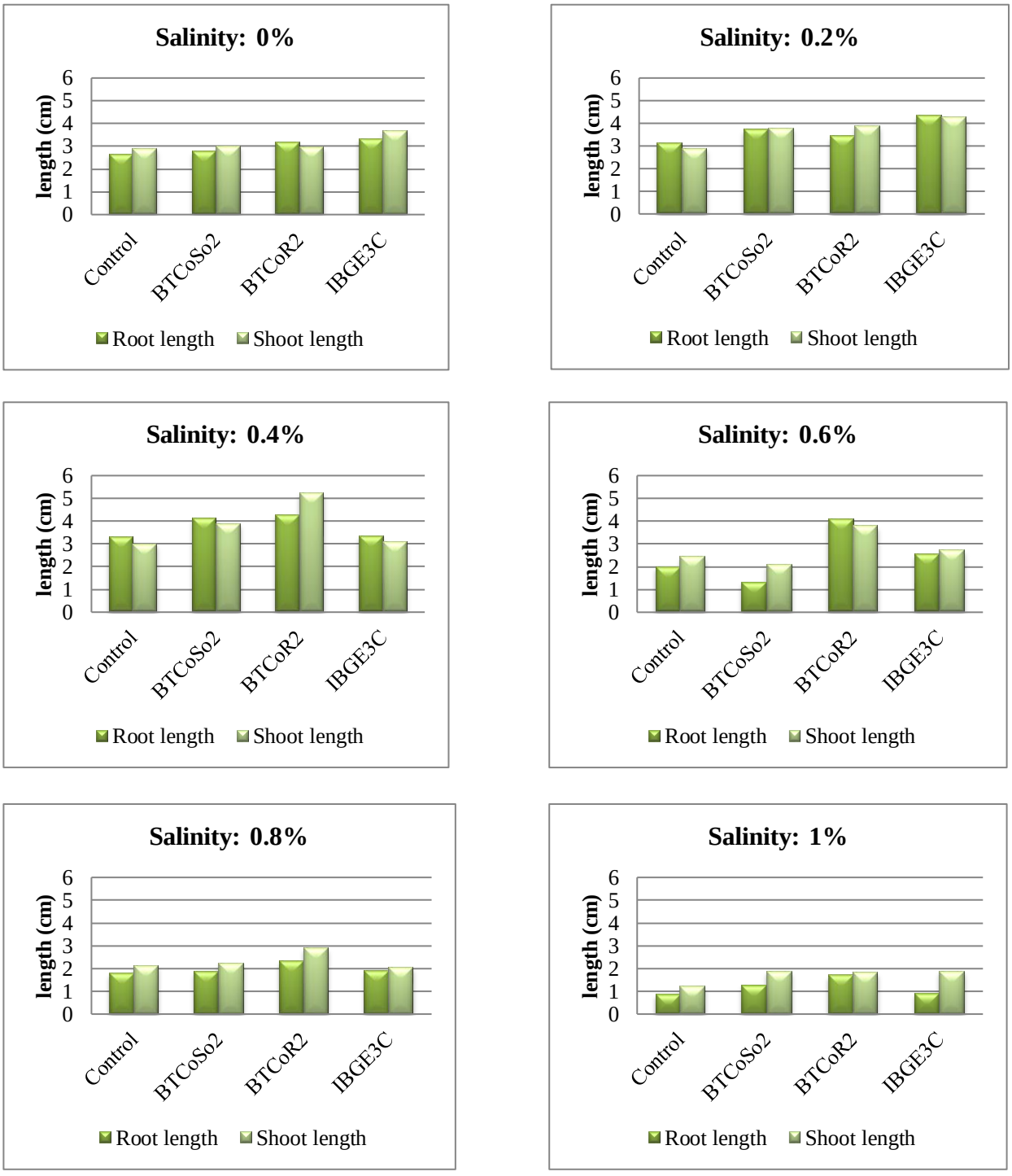


Figure 9: Root and shoot length (cm) variation of bacteria inoculated rice seeds in different salinity level (BRRI dhan 29)

Table 8: Effects of bacteria inoculation on root and shoot length of rice seedlings at seven days after inoculation (BRRI dhan 29)

Treatment	**Salinity level (%)**	**Seedling Growth**	
		Root length (cm)	**Shoot length (cm)**
No Bacteria	0	2.66	2.91
	0.2	3.13	2.89
	0.4	3.29	3.01
	0.6	1.98	2.43
	0.8	1.79	2.11
	1	0.87	1.21
BTCoSo2	0	2.79	3.01
	0.2	3.75	3.79
	0.4	4.12	3.89
	0.6	1.33	2.12
	0.8	1.89	2.23
	1	1.26	1.88
BTCoR2	0	3.17	2.97
	0.2	3.42	3.89
	0.4	4.28	5.23
	0.6	4.08	3.78
	0.8	2.31	2.89
	1	1.69	1.83
IBGE3C	0	3.32	3.68
	0.2	4.35	4.27
	0.4	3.33	3.09
	0.6	2.54	2.71
	0.8	1.91	2.03
	1	0.92	1.89

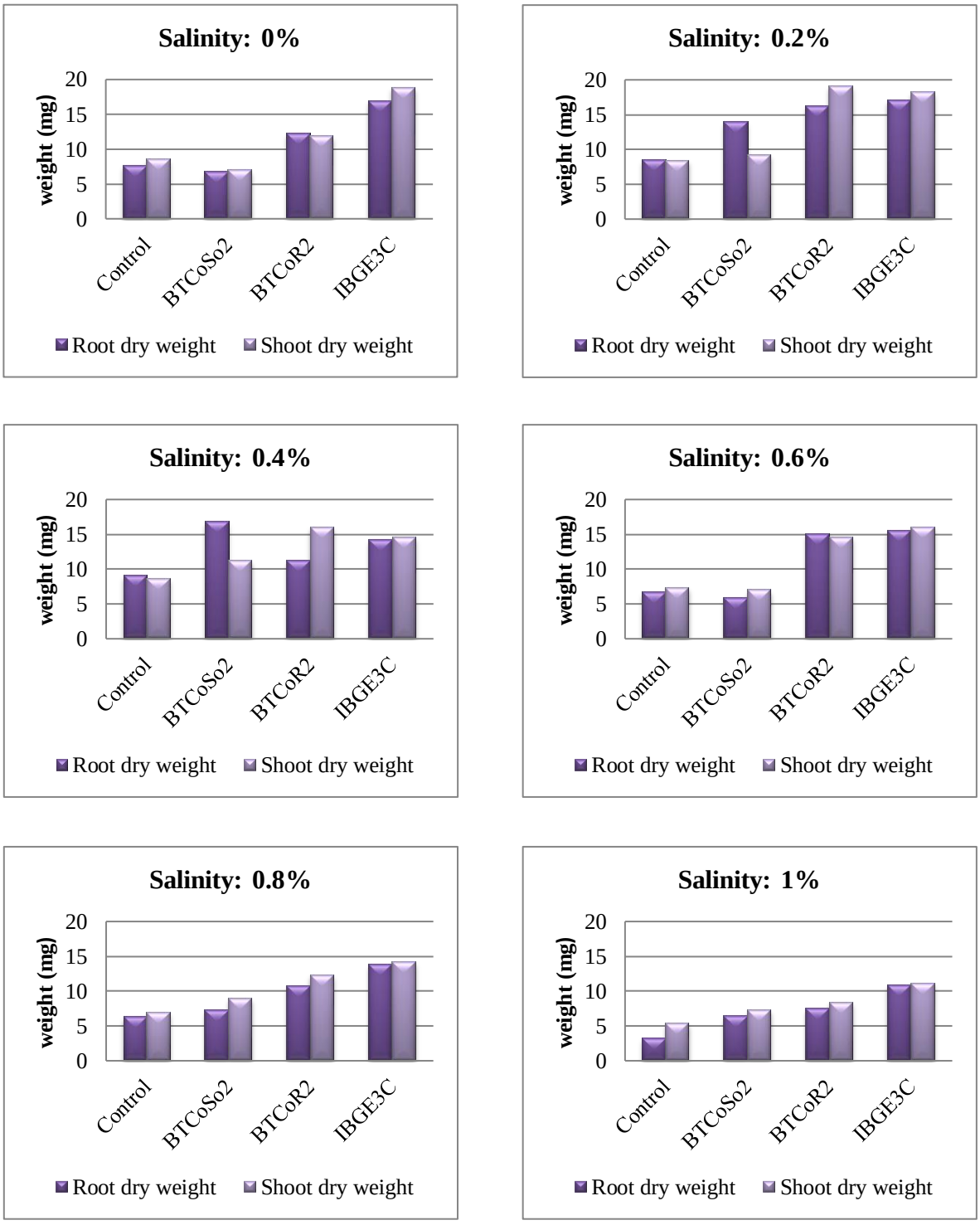


Figure 10: Root and shoot dry weight (mg) variation of bacteria inoculated rice seeds in different salinity level (BRRI dhan 29)

Table 9: Effects of bacteria inoculation on root and shoot dry weight of rice seedlings at seven days after inoculation (BRRI dhan 29)

Treatment	**Salinity level (%)**	**Seedling Growth**	
		Root dry weight(mg)	**Shoot dry weight (mg)**
No Bacteria	0	7.65	8.55
	0.2	8.51	8.42
	0.4	9.01	8.62
	0.6	6.62	7.39
	0.8	6.29	6.93
	1	3.22	5.39
BTCoSo2	0	6.69	7.01
	0.2	13.95	9.21
	0.4	16.81	11.22
	0.6	5.92	6.98
	0.8	7.21	8.95
	1	6.38	7.25
BTCoR2	0	12.18	11.81
	0.2	16.25	19.11
	0.4	11.19	16.02
	0.6	15.05	14.59
	0.8	10.81	12.31
	1	7.51	8.42
IBGE3C	0	16.95	18.89
	0.2	17.02	18.21
	0.4	14.18	14.57
	0.6	15.50	16.09
	0.8	13.82	14.23
	1	10.91	11.18

4.5 Effect of bacterial isolates on Seed germination in Petri dish (BINA dhan 10)

Root and shoot length along with their respective dry weight was recorded after seven days on PGPB inoculation. Highest root length was observed in IBGE3C at 0.2% salinity and lowest root length was observed in control at 1% salinity. Similarly, highest shoot length was observed in BTCoR2 at 0.2% salinity and lowest shoot length was recorded in control at 1% salinity.

In addition, IBGE3C produced the highest root dry weight at 0.2% salinity and lowest root dry weight was observed in control at 1% salinity. Also, highest shoot dry weight was produced by IBGE3C at 0.2% salinity and lowest shoot dry weight was recorded in control at 0.8% salinity.

Figure 11: Assessment of BINA dhan 10 rice seedling in Petri dish (from left to right: 0%, 0.2%, 0.4%, 0.6%, 0.8% and 1% NaCl (w/v)

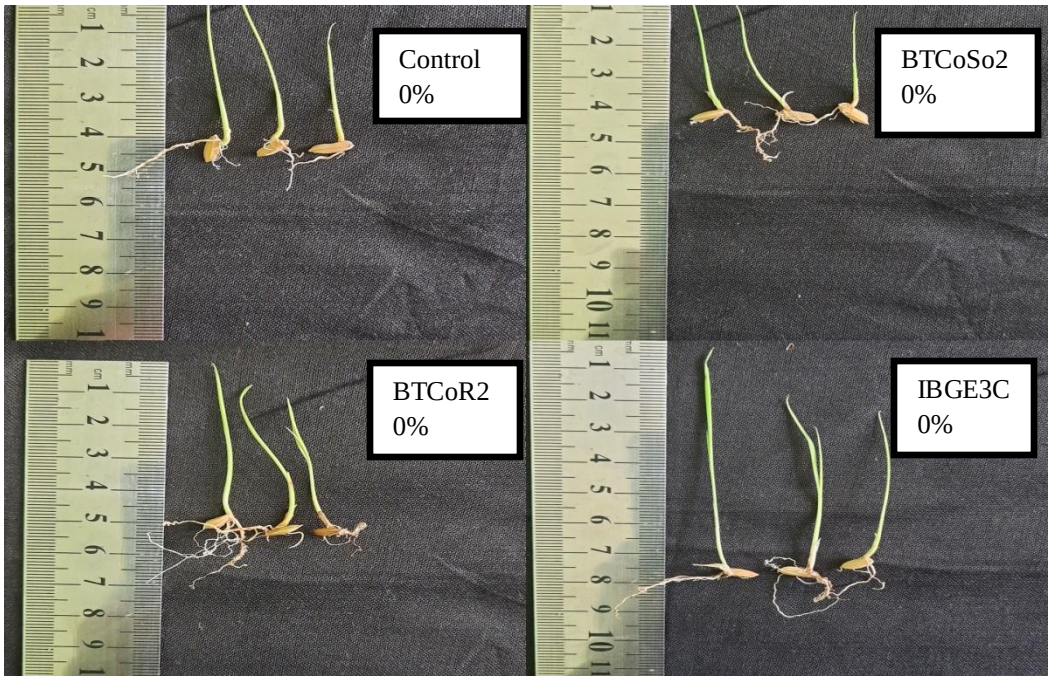
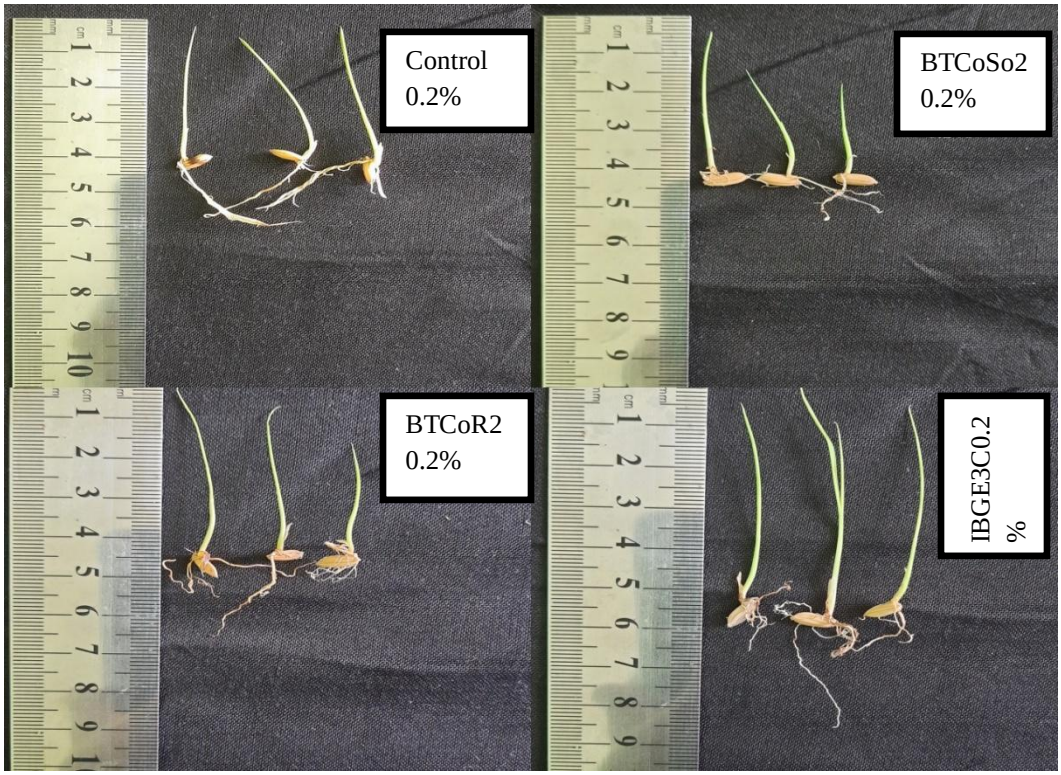


Figure 11: Continued…



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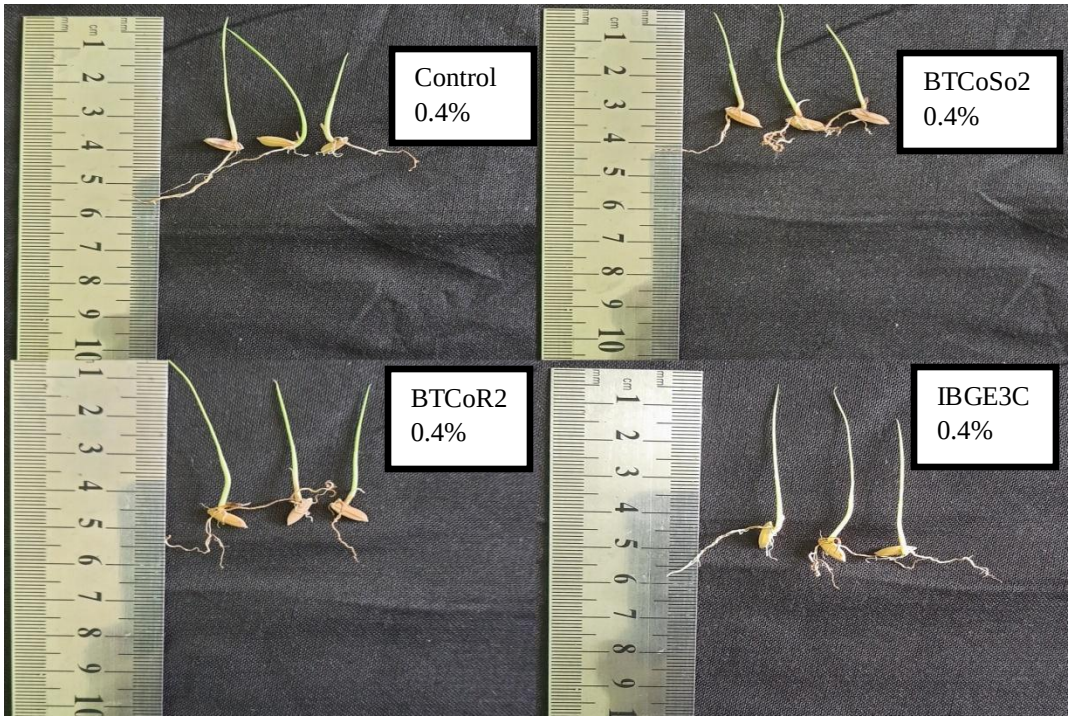

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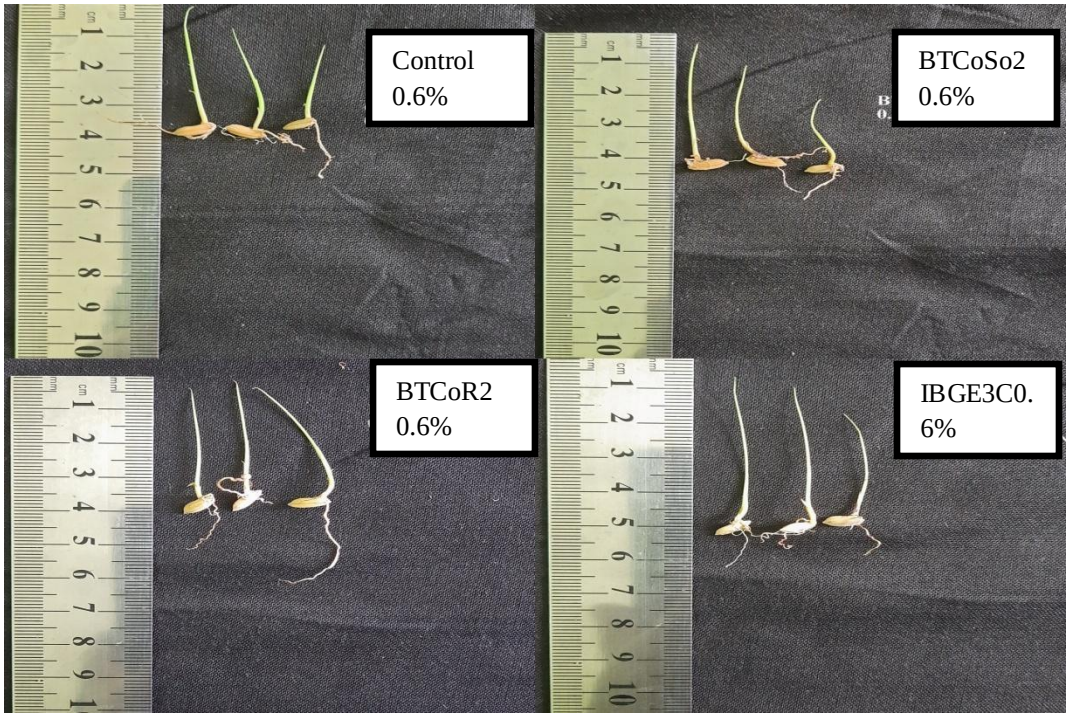

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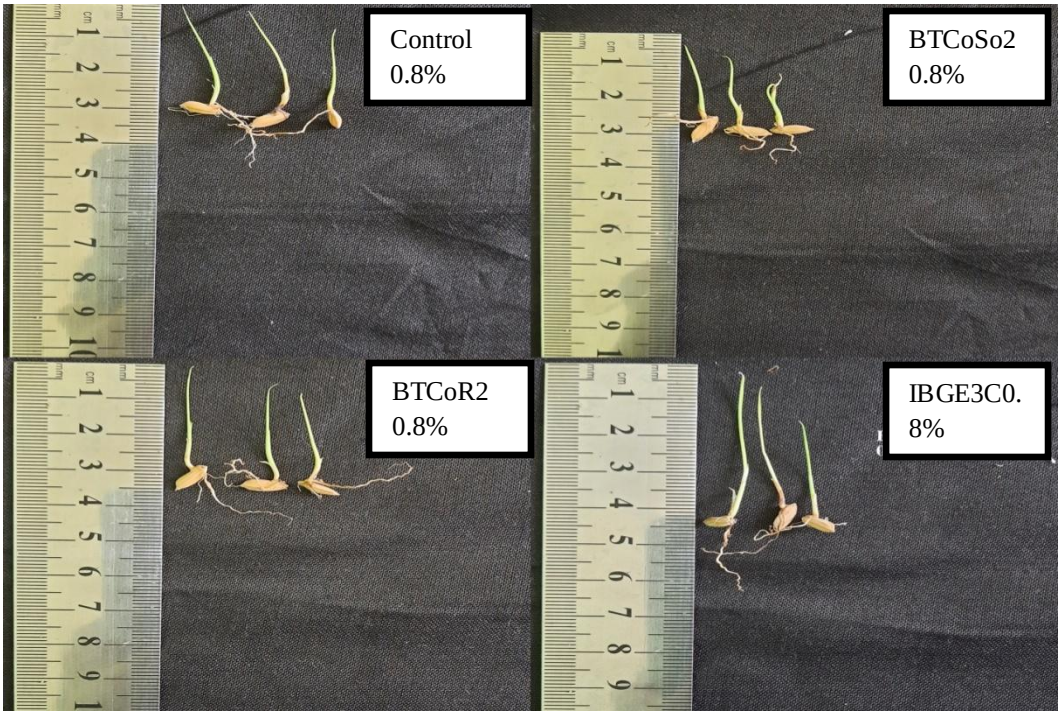

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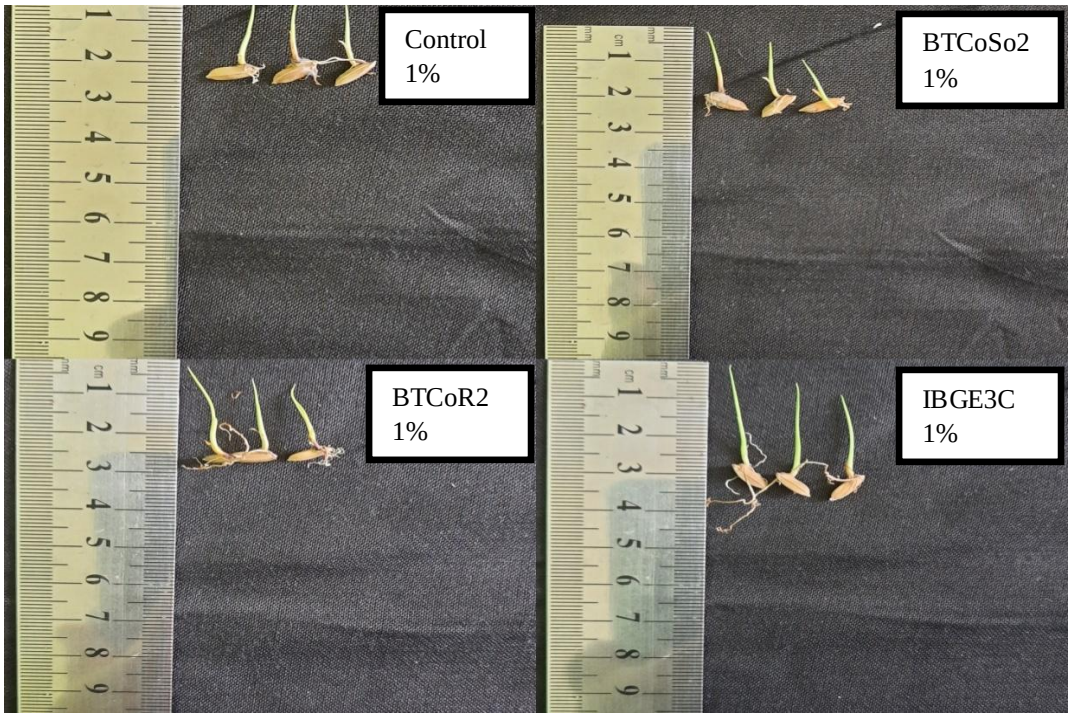

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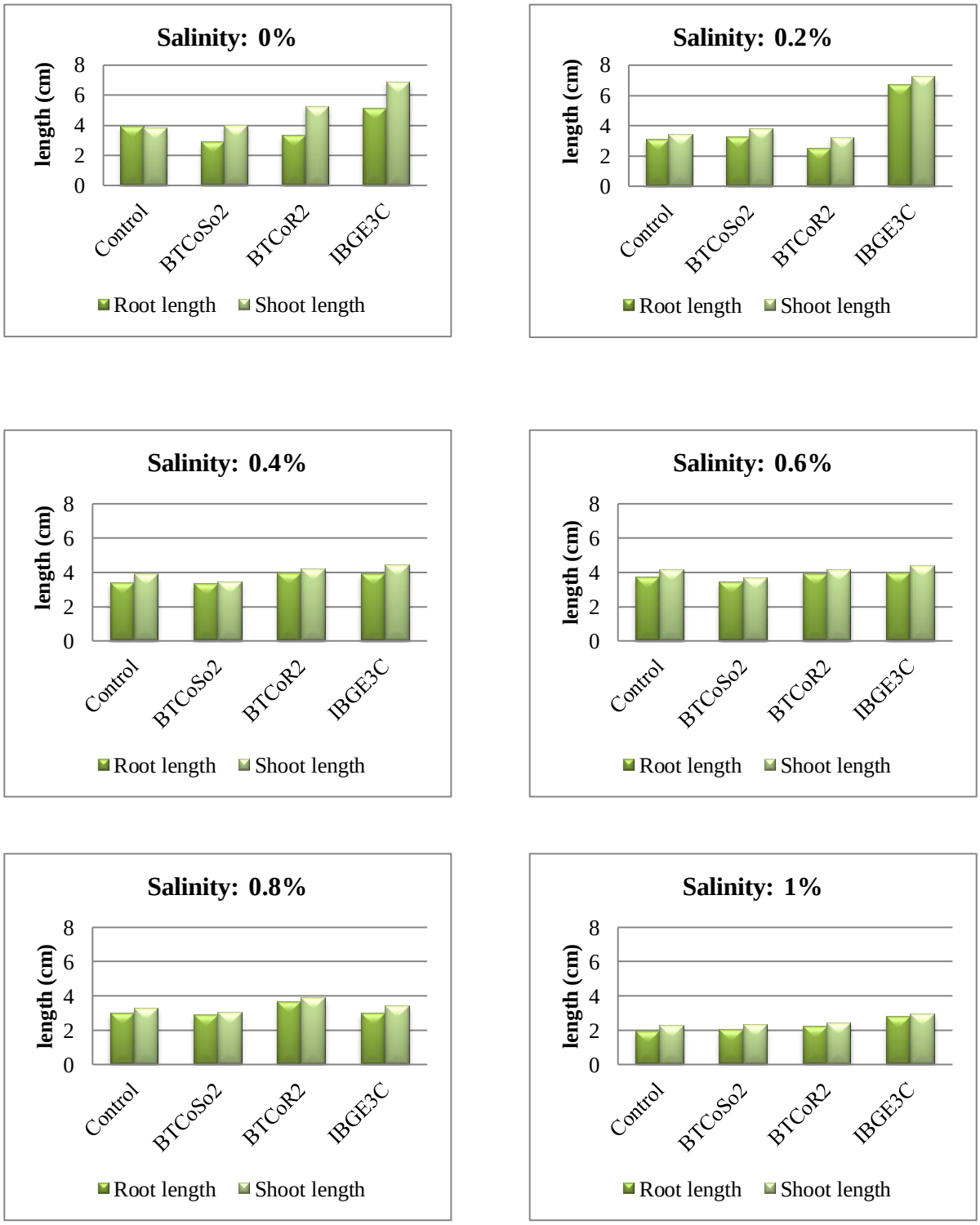

Figure 12: Root and shoot length (cm) variation of bacteria inoculated rice seeds in different salinity level (BINA dhan 10)

Table 10: Effects of bacteria inoculation on root and shoot length of rice seedlings at seven days after inoculation (BINA dhan 10)

Treatment	**Salinity level (%)**	**Seedling Growth**	
		Root length (cm)	**Shoot length (cm)**
No Bacteria	0	3.91	3.78
	0.2	3.12	3.43
	0.4	3.39	3.87
	0.6	3.71	4.16
	0.8	2.98	3.26
	1	1.92	2.23
BTCoSo2	0	2.92	3.97
	0.2	3.27	3.76
	0.4	3.32	3.43
	0.6	3.41	3.65
	0.8	2.87	3.01
	1	2.02	2.31
BTCoR2	0	3.31	5.21
	0.2	2.49	3.22
	0.4	3.95	4.21
	0.6	3.91	4.14
	0.8	3.66	3.87
	1	2.19	2.43
IBGE3C	0	5.08	6.89
	0.2	6.67	7.22
	0.4	3.91	4.43
	0.6	3.98	4.39
	0.8	2.97	3.41
	1	2.77	2.93

Figure 13: Root and shoot dry weight (mg) variation of bacteria inoculated rice seeds in different salinity level (BINA dhan 10)

Table 11: Effects of bacteria inoculation on root and shoot dry weight of rice seedlings at seven days after inoculation (BINA dhan 10)

Treatment	**Salinity level (%)**	**Seedling Growth**	
		Root dry weight(mg)	**Shoot dry weight(mg)**
No Bacteria	0	17.15	16.97
	0.2	16.29	16.88
	0.4	17.21	18.93
	0.6	18.25	20.21
	0.8	12.39	11.93
	1	10.82	13.32
BTCoSo2	0	13.33	15.02
	0.2	14.95	14.21
	0.4	15.01	16.18
	0.6	20.18	21.92
	0.8	17.93	19.25
	1	13.91	14.21
BTCoR2	0	20.92	22.52
	0.2	19.91	21.23
	0.4	15.21	22.23
	0.6	18.94	21.18
	0.8	12.21	14.37
	1	11.18	12.29
IBGE3C	0	24.02	27.01
	0.2	26.18	27.21
	0.4	14.16	23.93
	0.6	16.21	21.17
	0.8	15.34	23.19
	1	13.92	12.24

4.6. DNA extraction and PCR product purification

DNA extraction and PCR product purification of three bacterial isolates, BTCoSo2, BTCoR2 and IBGE3C were done for 16SrRNA gene sequencing for the identification and phylogenetic analysis of those bacteria.

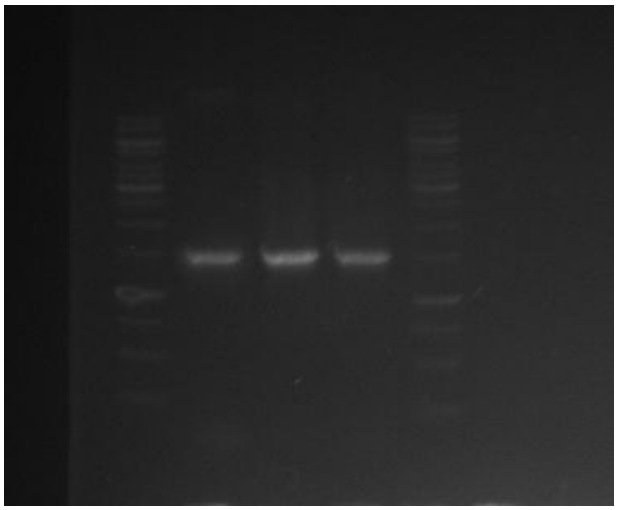

Figure 14: Gel electrophoresis photograph of purified PCR products of bacterial isolates (10µl/lane, 1% agarose, 0.05% TBE, electrophoresis time: 50 minutes; 1=ladder, 2=BTCoSo2, 3=BTCoR2, 4=IBGE3C, 5=ladder)

4.7. DNA sequencing and molecular characterization

IBGE3C bacteria showed comparatively better results and the PCR product was then sequenced. The strain exhibited 100% sequence homology with *Brevibacterium sediminis* IBGE3C strain and the sequence ID IS MZ573246.1

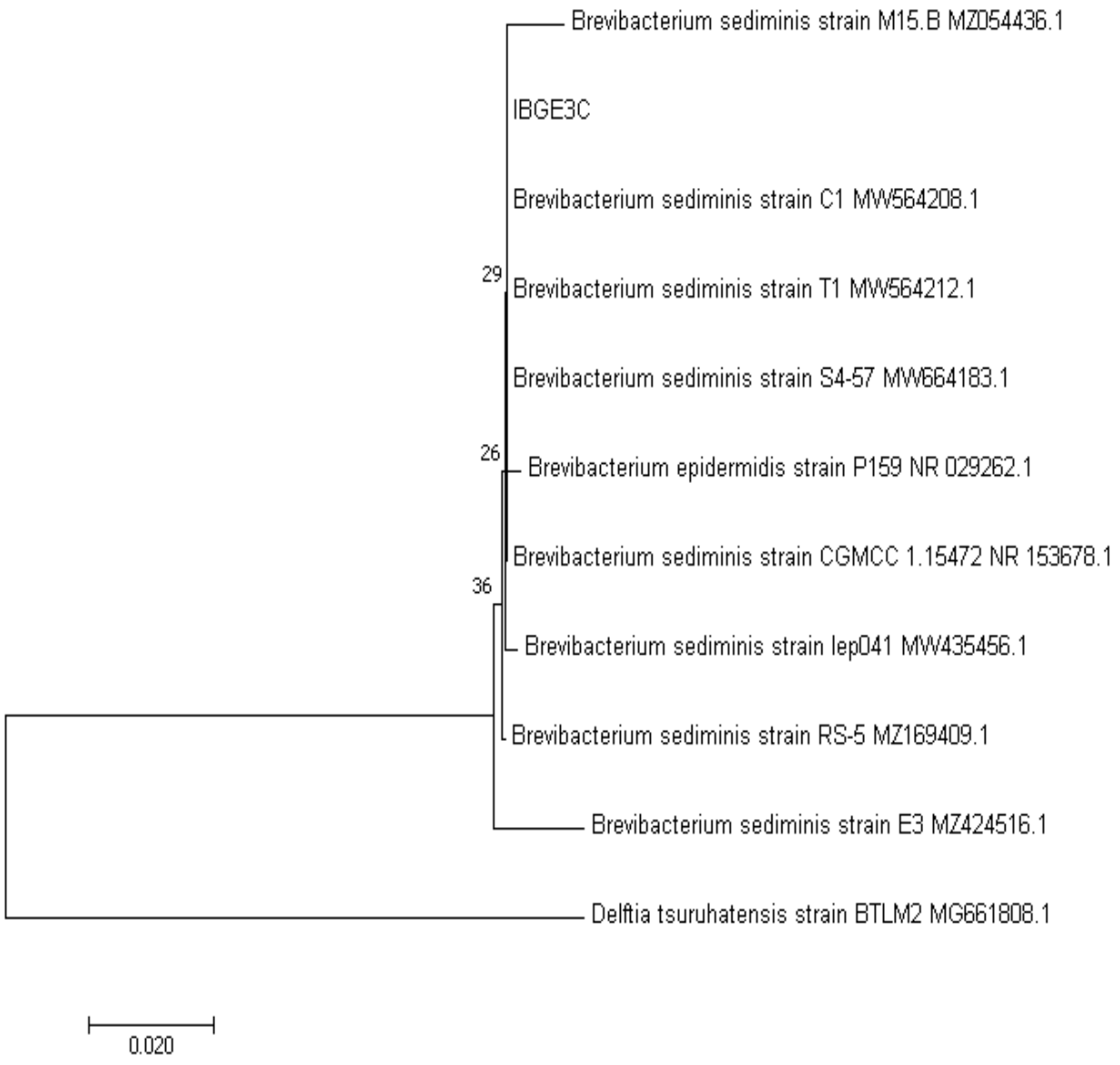

Figure 15: The Phylogenetic tree of *Brevibacterium sediminis* strain IBGE3C 16S ribosomal RNA gene

CHAPTER V

DISCUSSIONS

To ensure higher crop productivity by an environment-friendly salinity management, application of the PGPR is a promising biotechnological approach. This study aimed to isolate, identify and evaluate the novel rice probiotic bacteria, and evaluate their performances on seed germination and seedling growth of rice. A total of 41 bacteria isolated from the rice plants cultivated in the saline soils of Bangladesh and they were screened for salinity tolerance. Among them, three isolates viz. BTCoSo2, BTCoR2 and IBGE3C displayed higher tolerance to salinity up to 12% of NaCl (w/v) in the culture medium. These bacterial isolates further investigated for their effects on seedling growth of two popular varieties of rice differing from salinity tolerance. The findings of the research are discussed in this section with relevant literature.

5.1 Isolation and salinity tolerance performances of rice probiotics

Salinity is a big problem in crop production in the Southern districts of Bangladesh. The situation is aggravating due to the impacts of climate change. Salinity poses a serious threat to future food security of the country. This study isolated 41 rice-associated bacteria collected from salt affected areas in Chattogram, Noakhali, Lakshmipur and Cox's Bazar districts of Bangladesh. Bioassay revealed that these bacteria highly differed in salinity tolerance in vitro. Interestingly, three of them BTCoSo2, BTCoR2 and IBGE3C displayed tolerance up to 12% of salinity. Salinity tolerant bacteria from the salt adapted plants and soils have previously been reported in Kearl et al., (2019); Ansari et al., (2019); Sharma et al., (2021). However, this study for the first time isolated very high salinity tolerant bacteria associated with rice cultivated in the salinity

affected areas of Bangladesh. Enhancement of salinity tolerance in crop plants by the application of the PGPB has been reported earlier. To be effective in enhancing salinity tolerance by the plant probiotic bacteria, the isolate must be salinity tolerant. Therefore, the findings of this experiment of isolation of salinity tolerant PGPB from rice is encouraging for their evaluation in growth performances in rice. The mechanisms of salinity tolerance in bacteria have been reported in Kumar et al., (2020).

5.2 Effects of bacteria on rice seedling in Petri dish

Three salt stress tolerant PGPB isolated from rice remarkably improved the root and shoot growth of rice. Among two varieties tested, the PGPB isolates showed better performances in seedling growth of salt-tolerant rice variety BINA dhan 10 (Figure 11, 12 & 13) compared to salt-susceptible rice variety BRRI dhan 29 (Figure 8, 9 & 10). Enhancement of salt tolerance in rice and other crop plants by the application of the PGPB have been reported in Nakbanpote et al., (2014); Shultana et al., (2020); Wang et al. (2021); Chopra et al. (2020). Also, the growth of *Brevibacterium sediminis* in nutrient agar medium with up to 20% of NaCl (w/v) along with the optimum growth at 3.3% NaCl (w/v) have been previously reported in Chen at al. (2016). In the current study, we observed the optimum growth of IBGE3C at 10% NaCl (w/v) (Figure 5) and salinity tolerance level up to 12% of NaCl (w/v) (Figure 6) on nutrient broth agar medium. To add, improvement on rice seedlings by the inoculation of isolated PGPB through several physical parameters has been achieved through our research. In salt-susceptible BRRI dhan 29 at 1% salinity, inoculation with highly salt tolerant IBGE3C displayed 1.06% and 1.56% increase in the root length and shoot length respectively compared to the control line. Similarly, root dry weight and shoot dry weight was increased by 3.38% and 2.07% respectively by the treatment of the same isolate compared to control. Moreover, in salt-tolerant BINA dhan 10 at 1% salinity,

IBGE3C offered 1.44% and 1.31% increase in root and shoot length respectively. To add, although root dry weight was increased by 1.28%, shoot dry weight was decreased by 1.08% in the same condition. This study for the first time demonstrated that very high salt tolerant PGPB isolates native to rice plants cultivated in salt affected areas enhanced seedling growth of rice under varying levels of salinity. These findings are interesting clues for further investigation on performances and mode of action of these bacterial isolates in promoting growth and salt tolerance in rice in the field conditions in the salinity affected regions of Bangladesh. Although the mechanisms of salinity tolerance and growth promotion of plants by the application of the PGPB have not precisely been elucidated, however, a large body of literature is available on this aspect (Chopra et al., 2020; Chatterjee et al., 2018; Wang et al., 2021). Singh & Jha (2016); Vurukonda et al. (2016) has been previously demonstrated that PGPB minimize the effects of salinity by retaining required ratio of Na^+/ K^+ through excreting exopolysaccharides (EPS) that consequently secure their survival under salt stress. Similar to other PGPB, *Brevibacterium* sp. are able to produce high amounts of ACC deaminase and it has been found that inoculation of plants with ACC deaminase producing bacteria produce longer roots (Belimov et al., 2007). Kim et al. (2005) showed that high production of antioxidant enzymes reduces the generation of hydrogen peroxide under salt stress. Qin et al. (2016) reported that PGPB directly helps plant growth by producing IAA. Moreover, the sodium ion (Na^+) binding capacity of PGPB maintains cellular turgidity and defends chloroplast from adverse impacts of salinity and thus increase photosynthesis, chlorophyll synthesis and plant growth under salt stress (Kang et al., 2014; Del et al., 2011). In this study, the isolate IBGE3C displayed the best performances in terms of salinity tolerance and seedling growth of rice.

5.3 DNA sequencing and molecular identification of the bacteria

The 16S rRNA is a gold standard and convenient molecular method for molecular identification of the bacteria. Therefore, best performer rice probiotic bacterial isolate, IBGE3C was identified as *Brevibacterium sediminis* as it showed 100% sequence homology to the reference genome published in the NCBI database (accession no. MZ573246). Although salinity tolerant *Brevibacterium* spp. have been discovered as salinity tolerant bacteria from some soils (Chen et al., 2016; Ansari et al., 2018: Wang et al., 2021) and plant sources (Chopra et al., 2020; Siddikee et al., 2010), this study for the first time discovered a highly salinity (12% NaCl w/v) tolerant *B. sediminis* strain IBGE3C from rice plants cultivated in the salt affected area (Lakshmipur) of Bangladesh. Further in silico and laboratory analyses of the whole-genome sequence of this salinity tolerant bacteria would result in interesting insights of its plant growth promoting and salinity tolerance mechanism that are needed for its practical application in rice.

CHAPTER VI

SUMMARY

The PGP bacteria are capable of enhancing plant growth under salt-stress. This study aimed to isolate, screen, and characterize rice growth promoting bacteria from rice plants cultivated in saline soils of Chattogram, Noakhali, Lakshmipur and Cox's Bazar districts of Bangladesh. A total of 41 bacteria were isolated and screened for their salinity tolerance. Three highly salinity tolerant rice associated bacteria were evaluated for their growth promoting effects on two cultivars of rice (BRRI dhan 29 and BINA dhan 10) under varying levels of salinity tolerance. Although both bacteria improved seedling growth of rice, these isolates showed better performances on BINA dhan 10 compared to BRRI dhan 29. The best performing salinity tolerant bacterium IBGE3C was identified as *B. sediminis* strain IBGE3C using 16S rRNA gene sequencing. This novel isolate needs further molecular and field studies for judging its suitability as a bioinoculant for production of rice in the saline soils of Bangladesh.

CONCLUSION

This study discovered three highly salinity tolerant bacteria viz. BTCoSo2, BTCoR2 and IBGE3C from the rice cultivated in salinity affected soils of Bangladesh. They generally improved seedling growth of rice varieties with varying levels of salinity tolerance, BRRI dhan 29 and BINA dhan 10. However, the effects of the bacteria were more pronounced in BINA dhan 10. Among the bacterial isolates, the best performing one, IBGE3C were subjected to molecular identification using 16S rRNA gene sequencing. Interestingly, 16S rRNA gene sequencing of IBGE3C displayed 100% sequence homology with *B. sediminis*. Discovery of an isolate of salinity tolerant and rice growth promoting *B. sediminis* is the first evidence in Bangladesh. A further study is needed for field evaluation of the performances of *B. sediminis* IBGE3C on rice in the saline soils of Bangladesh

CHAPTER VII

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APPENDIX

APPENDIX A: *Brevibacterium_sediminis* IBGE3C sequence

The sequence is: >*Brevibacterium_sediminis* IBGE3C

CTACACATGCAGTCGAACGCTGAGCCGACAGCTTGCTGTTGGTGGATGAGTGGCGAACGGG
TGAGTAACACGTGAGTAACCTGCCCCTGATTTCGGGATAAGCCTGGGAAACTGGGTCTAATA
CCGGATAYGACCAATCCTCGCATGAGGGTTGGTGGAAAGTTTTTCGATCGGGGATGGGCTCG
CGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTG
AGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCAGCGTGCGGGATGACGGCC
TTCGGGTTGTAAACCGCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAG
TACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTACGAGCGTTGTCCGGAATT
ATTGGGCGTAAAGAGCTCGTAGGTGGTTGGTCACGTCTGCTGTGGAAACGCAACGCTTAACG
TTGCGCGTGCAGTGGGTACGGGCTGACTAGAGTGCAGTAGGGGAGTCTGGAATTCCTGGTGT
AGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGACTCTGGGCTGT
AACTGACACTGAGGAGCGAAAGCATGGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA
TGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTCTCCGCGCCGTAGCTAACG
CATTAAGTGCCCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG
CTTGACATACACTGGACCGTTCTGGAAACAGTTCTTCTCTTTGGAGCTGGTGTACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TCGTTCTATGTTGCCAGCACGTGATGGTGGGAACTCATAGGAGACTGCCGGGGTCAACTCGG
AGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTTATGTCTTGGGCTTCACGCATGCTAC
AATGGCTGGTACAGAGAGAGGCGAACCCGTGAGGGTAAGCGAATCCCTTAAAGCCAGTCTC
AGTTCGGATCGTAGTCTGCAATTCGACTACGTGAAGTCGGAGTCGCTAGTAATCGCAGATCA
GCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCTCAAGTCACGAAAGTCG
GTAACACCCGAAGCCGGTGTCCCAACCCTTGGGACGGGCCTC

APPENDIX B: Growth of bacterial isolates at different salt concentration in nutrient broth agar medium

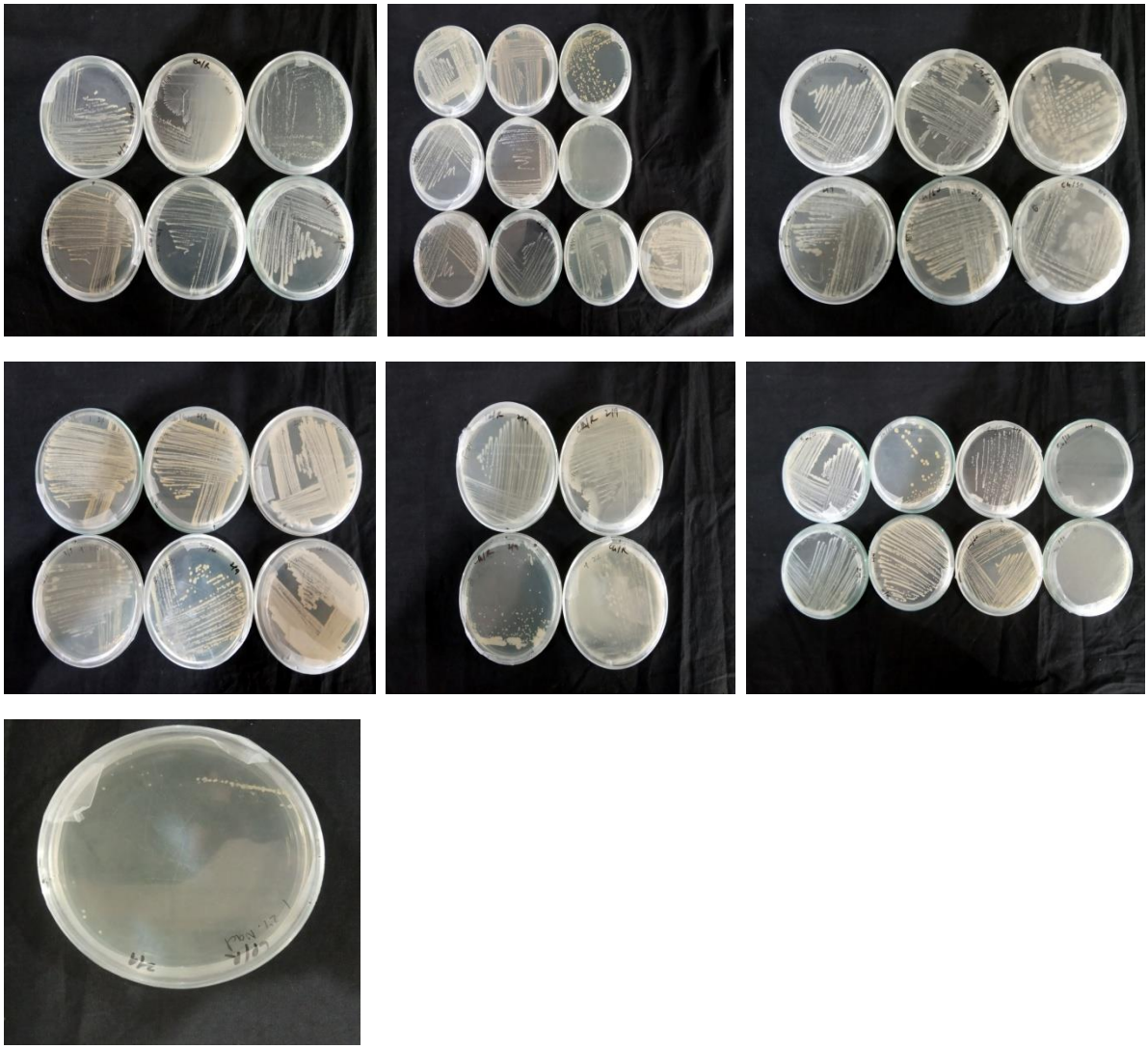

Figure B1: Growth of bacterial isolates in nutrient broth agar (NBA) medium with 2% NaCl (w/v)

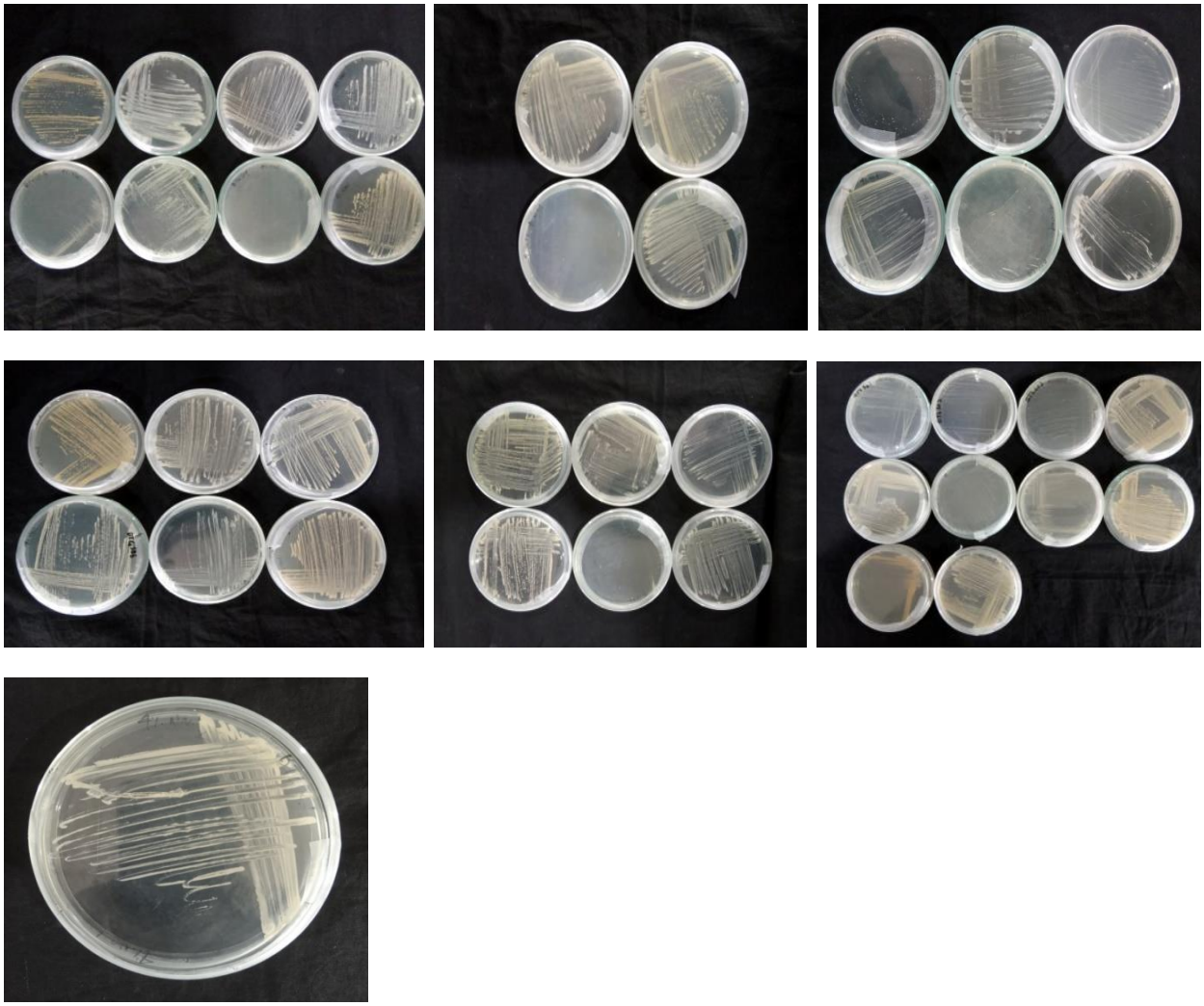

Figure B2: Growth of bacterial isolates in nutrient broth agar (NBA) medium with 4% NaCl (w/v)

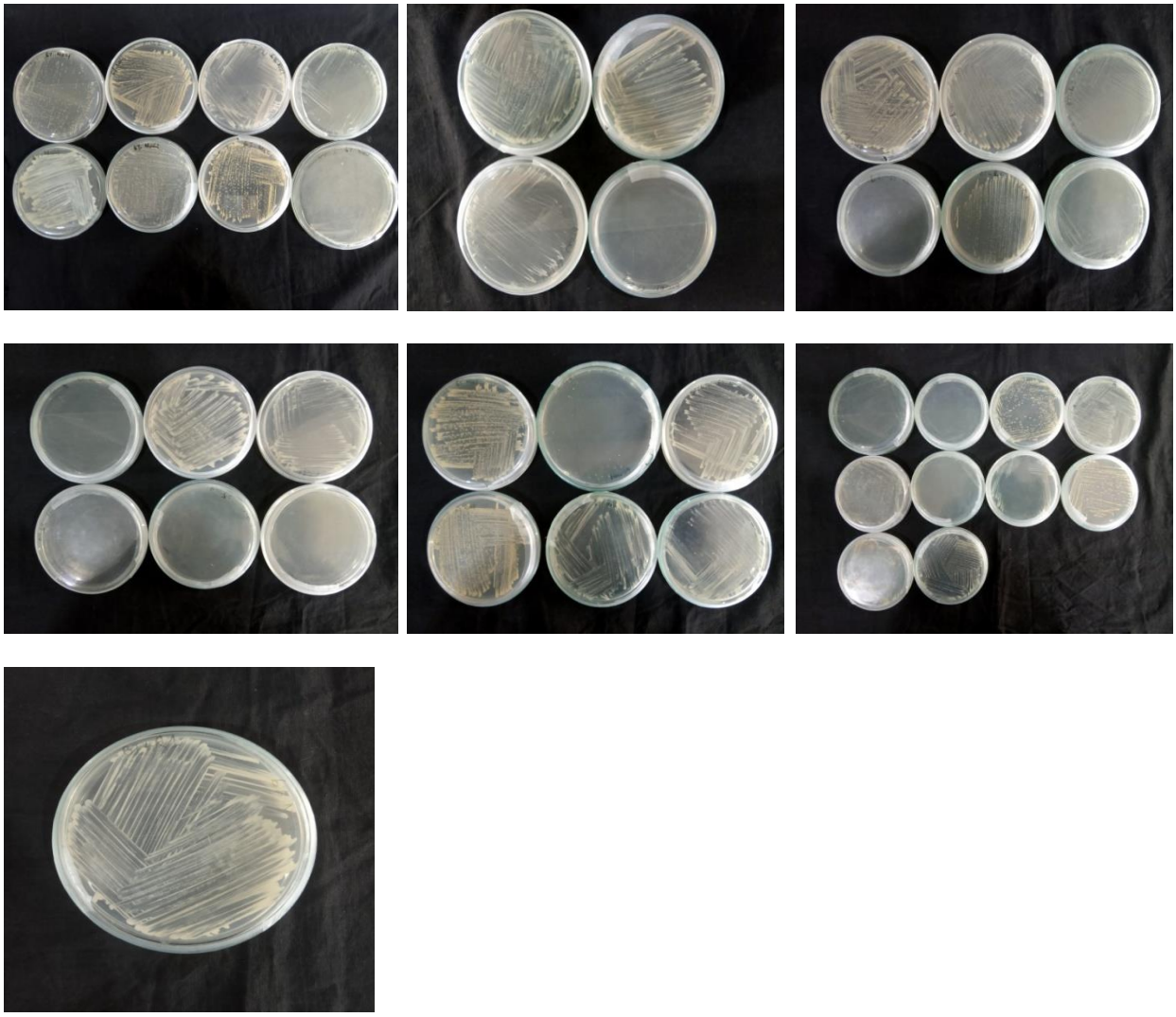

Figure B3: Growth of bacterial isolates in nutrient broth agar (NBA) medium with 6% NaCl (w/v)

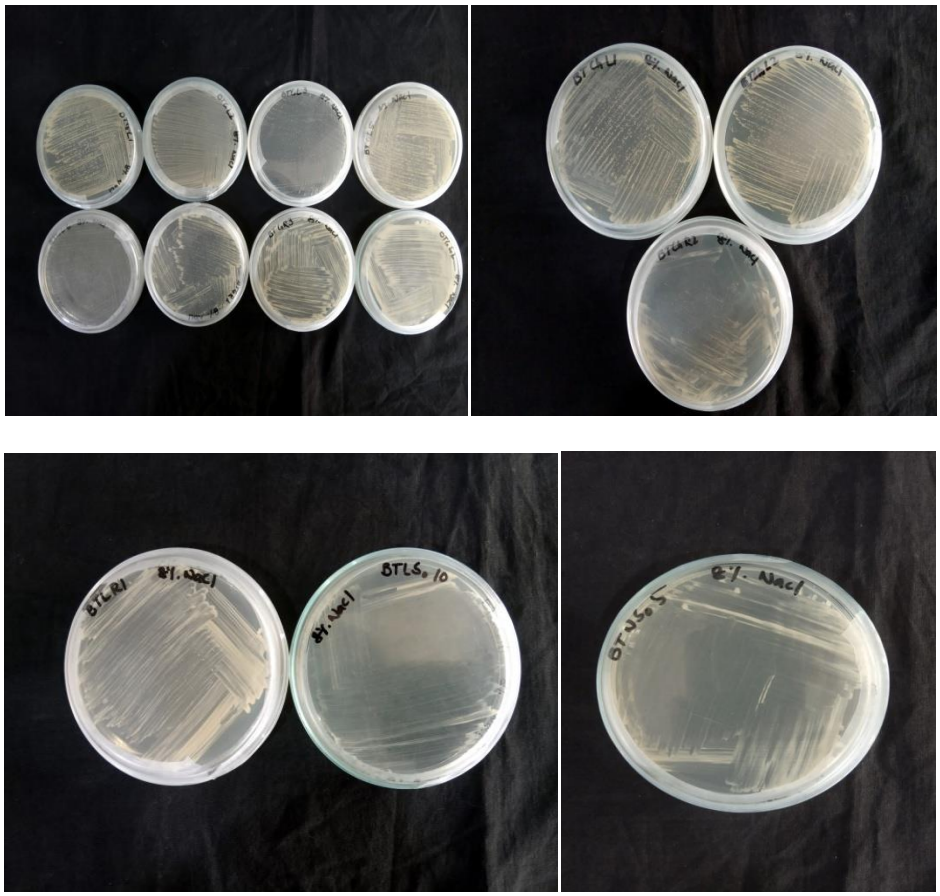

Figure B4: Growth of bacterial isolates in nutrient broth agar (NBA) medium with 8% NaCl (w/v)