Study of Siderophore Producing Microbes for their Tolerance Against Antibiotics and Metals.

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I hereby declare that the data presented in this Dissertation report entitled, "Study of Siderophore Producing Microbes for their Tolerance Against Antibiotics and Metals" is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Siddhi K. Jalmi and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report "Study of Siderophore Producing Microbes for their Tolerance Against Antibiotics and Metals" is a bonafide work carried out by Ms Geeta Ramesh Mestry under my supervision in partial fulfilment of the requirements for the award of the degree of M.Sc. in the Discipline of Botany at the School of Biological Sciences and Biotechnology, Goa University.

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CONTENT

PREFACE

Land degradation is a very notable environmental issue at the local, regional, and global levels for agriculture. Plants and other organisms are threatened by heavy metals accumulating in the soil due to various human activities such as mining. The rhizospheric system of the plant has diverse modes and efficient mechanisms to cope with various stresses by numerous root-associated microbes. The contaminated soil with metal and antibiotics is known to hamper plant growth productivity. Therefore, in the context of tackling these issues, the present study aims to identify promising metal and antibiotic-tolerant siderophore-producing bacteria which could potentially act as plant-growth-promoting bacteria (PGPB) under metal-contaminated soils. Siderophores are well-recognized iron-chelating agents produced by numerous microbes and are associated with the rhizosphere. These siderophore-producing microbes are eco-friendly and sustainable agents, which may manage plant stresses in degraded land.

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Ms. Geeta Ramesh Mestry

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ABBREVIATIONS USED

Entity	Abbreviation
Plant Growth-Promoting Rhizobacteria	PGPR
Lead-Tolerant-Plant-Growth-Promoting Rhizobacteria	ZTPGPR
Zinc-Tolerant Plant Growth-Promoting Rhizobacteria	LTPGPR
Zinc Tolerant Bacteria	ZTB
Lead Tolerant Bacteria	LTB
Siderophore-Producing Microbes	SPM
Chrome Azurol Sulfonate Assay	CAS

ABSTRACT

Plant growth performance is adversely affected by metal contamination in the soil, but prior research has shown that bacteria that produce siderophores and are tolerant to metals can assist plants to overcome metal stress. The research aimed to obtain some siderophore-producing strains which are tolerant to Pb and Zn and study their plant growth-promoting capabilities under Pb and Zn-contaminated soil. The present work was designed to isolate the siderophore-producing bacteria from the rhizosphere of *Oryza sativa*. The obtained isolates have been screened for siderophore production using Chrome Azurol Sulfonate assay (CAS). The highest siderophore-producing bacteria were B1 and B2 showing the highest halo-colony ratio of 1.97 and 2.59 respectively. B1 and B2 siderophores were characterized as catecholate types with maximum absorbance at 495 nm. These superior strains were screened using pot experiments to study their growth-promoting ability in the Lead (Pb) and Zinc (Zn) contaminated soil. There was an increase in the shoot length of 13% and 16% when B1 and B2 were applied in Pb-contaminated soil respectively, while a 24% and 11% increase in the shoot length was observed when B1 and B2 respectively were applied to Zn-contaminated soil.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Rice (*Oryza sativa* L.) is the world's most significant food crop, providing a staple diet for almost 3 billion people, or roughly half of the world population. Rice is a staple food crop in many countries, including India. India as reported by the Ministry of Information and Broadcasting, Government of India in 2022 in AKAM Series is the second largest producer of rice globally, with a record production of 121.46 million tonnes in the 2020-21 crop year.

Rice is the predominant stable food crop of Goa. Presently in Goa, rice is cultivated over an area of 42,973 ha, with a production of 1,55,818 m annually. The average productivity of the crop is about 3,399 kg/ha. High-yielding rice varieties such as *Jaya* and *Jyoti* are predominantly cultivated in the state of Goa. The rice variety *Korgut* is popularly cultivated in the *Khazan* lands of Goa due to its high salinity tolerance (Bhonsle & Sellappan, 2010).

The cultivation of rice is prevalent on a global scale however, its development and efficiency are hindered by stress caused by high levels of metallic elements. Mining has been crucial for Goa's economy, which was practised by an open-cast method which necessitates the removal of overburden overlying the iron ore formations. On average about 2.5 to 3 tonnes of mining waste has to be excavated to produce a ton of iron ore. Most mining areas are in Bicholim, Sattari, Sanguem and Quepem talukas. The maximum area under mining is in Sanguem talukas followed by Bicholim, Sattari and Quepem. Heavy rainfall during monsoons causes surface runoffs of heavy metals and siltation from mining sites which affect the agricultural fields and rivers in Goa resulting in soil infertility, crop loss, and reduced agricultural yield. Farmers have reported a decrease in crop yields and the inability to grow crops due to the dust generated by mining activities. Villages in Bicholim Taluka have their fields rendered useless, which are choking with mining rejects (Talule & Naik, 2014). Mining also affects microorganisms by providing toxic minerals hindering their activity (Nayak, G., 1998).

Introduction

The fostering of agricultural sustainability relies completely on soil, as it is a precious natural asset. Soil metal pollution from human activities is a significant worldwide issue and threat to mankind (Adam, 2002). The operations of mining, smelting, and using metals and metal-containing compounds in diverse applications, including agriculture, are the main causes of heavy metal contamination and exposure, which poses a serious risk to the environment and human health. Many heavy metals in ppm quantities are necessary as trace elements; but, at higher concentrations, they become toxic elements (Mihdhir et al. 2016). Some heavy metals are essential micronutrients for plants, microorganisms and organisms at higher trophic levels such as zinc, iron, copper, and nickel. Other heavy metals for example mercury, cadmium, lead and arsenic are non-essential elements (Ali et al., 2019). Lead (Pb), Copper (Cu), Cadmium (Cd), Chromium (Cr), Mercury (Hg), Zinc (Zn), Arsenic (As) and Nickel (Ni) are the most common heavy metals found in contaminated soils (Fashola et al., 2016; Shahid et al., 2017; Ali et al., 2019).

Lead (Pb) is a persistent toxic heavy metal for humans, which is classified as a potential human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC,1993) (Francek, 1992; Silbergeld et al., 2000). Lead is a major anthropogenic contaminant, released into the environment mainly from mining, smelters and industrial production discharge (Small et al., 1995; Caussy et al., 2003; Deng et al., 2004; Marchiol et al., 2004). Lead in soil is primarily introduced through atmospheric deposition, including emissions from vehicles, combustion of fuel and waste. Various plants, such as rice (*Oryza sativa*) and vegetables, can accumulate lead from different sources, including absorption from soil contaminated with lead (Verma and Dubey, 2003; Yoon et al., 2006; Liu et al., 2013). Studies have reported that lead contamination from mining activities can potentially endanger the health of residents through the food chain (Zhang et al., 1998; Capdevila et al., 2003; Yang et al., 2004; Zhuang et al., 2009).

Introduction

Zinc (Zn) is one of the most crucial elements required for the development of plants. Zinc is a trace element considered a micronutrient as it is required in minute amounts by plants (Sharma et al., 2013). Soil mainly contains zinc in large concentrations but in inactive forms in minerals like franklinite ($ZnFe_2O_4$), willemite (Zn_2SiO_4), hopeite $Zn_3(PO_4)_2$, sphalerite (ZnS), smithsonite ($ZnCO_3$), Znite (ZnO), and sphalerite (ZnS) (Broadley et al., 2007). Less than 0.6 ppm indicates a deficiency in zinc in the soil, while more than 1.2 ppm suggests toxicity. The critical value of zinc in the soil ranges from 0.6 to 1.2 ppm, or mg/kg of soil. However, the concentration of zinc in plants can reach up to 20-300 ppm. Still, only a minute level is in readily available form as the solubility of Zn is very low in the soil as compared to other nutrients (Athokpam et al., 2018). Soil contaminated with zinc exerts detrimental effects on both plants and the soil microbiome; nevertheless, these soils exhibit an abundance of zinc-tolerant plant growth-promoting rhizobacteria (ZTPGPR) (Redmile-Gordon & Chen, 2017).

A study reported that Fe deficiency is a common phenomenon in heavy metal-contaminated soil (Leskova et al., 2017). Iron (Fe) is an essential element for the growth of almost all living microorganisms because it acts as a catalyst in enzymatic processes, oxygen metabolism, electron transfer, and DNA and RNA synthesis. Siderophores are low molecular weight (200-2000Da) secondary metabolites produced by microbes and plants under iron-deficient conditions, to supply iron to the organism. The role of siderophores is primarily to scavenge Fe. Still, they also form complexes with other essential elements (i.e. Mo, Mn, Co and Ni) in the environment and make them available for microbial cells (Bellenger *et al.,* 2008; Braud *et al.,* 2009a). There are different classes of siderophores depending on the characteristic functional group such as hydroxamate, catecholate and carboxylates. More than 500 different types of siderophores are known, of which 270 have been structurally characterized (Boukhalfa *et al*., 2003).

Introduction

Investigating the possibility of employing siderophores in metal bioremediation has drawn more attention in recent years. Bioremediation as a natural process involves the utilization of living organisms or enzymes for detoxifying heavy metals from the environment, which has garnered significant interest. This method is superior to conventional chemical processes with the added benefit of preserving soil fertility (Singh et al., 2009; Saranraj and Stella 2012). However, it is a gradual process and may require numerous years for the rehabilitation of metal-polluted soils to a standard, healthy condition. Circumstances might vary based on the responsiveness of metals and soil composition (Lira et al., 2020).

Phytoremediation is an eco-friendly remediation process regulated by crop plants (Lis-Balchin, 2002). Trees, bushes, and grasses, in association with microorganisms, mediate the contaminated environment (Abouelatta et al., 2020). The metal uptake capacities of plants are extremely crucial to this technique (Verma et al., 2020). Phytoremediation is a crucial sustainable technology with the capability to eliminate contamination from the soil such as organic pollutants, heavy metals, etc while diminishing the production of additional waste (Lira et al., 2020). Phytoremediation is eco-friendly, cost-effective, operationally viable, and relatively simple technology to execute as it does not necessitate costly apparatus or skilled personnel (Baliyan et al., 2022). Phytoremediation effectiveness can be enhanced through the interaction of metal-tolerant plant growth-promoting rhizobacteria, which enhance plant growth and maturation in the presence of metal-stress environments through various direct and indirect mechanisms (Tepe et al., 2006). These mechanisms encompass the phytohormonal production of cytokinins, gibberellic acid or indole acetic acid, ACC production under stress conditions, mineral solubilization through phosphorus and potassium, the production of antifungal compounds, and notably, the immobilization of heavy metals via exopolysaccharides (EPS) generation (Jazayeri et al., 2014).

1.2 AIM AND OBJECTIVES

- To isolate siderophore bacteria from the rhizosphere soil of the *Jaya* rice variety growing near the mining area.
- To study the tolerance of siderophore-producing bacteria against antibiotics and metals [Lead (Pb) and Zinc (Zn)].
- To study the role of siderophore-producing metal-tolerant bacteria in enhancing plant growth under metal-contaminated soils.

1.3 HYPOTHESES/ RESEARCH QUESTION

- Can Siderophore-producing bacteria exhibit varying levels of tolerance to antibiotics and metals (such as lead and zinc)?
- Does metal-tolerant siderophore-producing bacteria able to positively impact plant growth under metal-contaminated soils?

1.4 SCOPE

- The study will focus on isolating siderophore-producing bacteria from the rhizosphere soil of the *Jaya* rice variety growing in fields near the mining area.
- Antibiotic and metal tolerance assays will be conducted to assess bacterial resilience for antibiotics and metals and their effect on plant growth in metal-contaminated soil.
- The studied bacteria can be future potential biofertilizers for crops growing in metal-contaminated areas.

CHAPTER 2: LITERATURE REVIEW

2.1 TRACE ELEMENT CONTAMINATION IN AGRICULTURAL LAND

A study reported that 6.24% of agricultural land shows trace elements (e.g., As, Cd, Cr, Cu, Hg, Pb, Zn, Sb, Co and Ni) exceeding legal limits (Tóth et al., 2016). In plants, for example, Zn and Pb contamination can contribute to altering the plant water balance (Igiri et al., 2018). Contamination is a threat to food security in the case of bioaccumulation (Kushwaha et al., 2017; Vardhan et al., 2019). Heavy metal toxicity is seen to influence plant growth, root elongation and seed germination (Kushwaha et al., 2017).

2.2 RICE AS A VITAL GLOBAL FOOD SOURCE

Rice is a major food source for 60% of the global population, especially in Asia. It is known to effectively absorb heavy metals and transport them to the grains more than other plants (Williams et al., 2007). The sensitivity of plants to heavy metals differs. Some species can survive and grow with a high level of heavy metals; they are termed hyperaccumulating plants and can accumulate up to 100 μg/g of each metal, such as Cd and Cu, and much higher concentrations of other metals (Ojuederie & Babalola, 2017). More than 500 taxa are considered hyperaccumulating plants. These species can be very useful for remediation purposes; however, it is not desirable to use them for edible crops in contaminated sites (Ojuederie & Babalola, 2017; Ancona et al., 2019).

2.3 TOXIC HEAVY METALS: IMPACT ON FLORA, FAUNA, AND AGRICULTURAL PRODUCTS

The soil contains a high concentration of heavy metals due to continuous human activities such as mine exploitation, the application of sewage water for irrigation, the use of agrochemicals enriched with metals, and the smelting of metals from ores contribute to an increase in the discharge and the quick spread of heavy metal pollution in human settlements and natural habitats, having a disastrous effect on agricultural products. The rise in heavy metals poisoning in the environment, detrimental to living organisms, is attributed to heightened

anthropogenic activity, rapid industrialization, and modern agricultural practices in recent decades (Singh et al., 2016). An elevation in the concentration of heavy metals in the soil poses various challenges for flora and fauna (Alengebawy et al., 2021).

Heavy metals are natural elements that have high atomic mass and density approximately 5 times greater than water (Tchounwou et al., 2012). Many heavy metal ions are essential as trace elements in ppm quantities, but at high concentrations, they turn into toxic elements (Mihdhir et al., 2016). Both essential and nonessential heavy metals, including mercury (Hg), chromium (Cr), copper (Cu), and zinc (Zn), can be highly toxic to plants when present in excess (Qadir et al., 2020; Khan et al., 2021). The common indicators of essential and non-essential heavy metals in plants encompass the reduction in root and shoot elongation, the number of tillers per plant, levels of chlorophyll and protein contents, and a rise in the production of reactive oxygen species (ROS), malondialdehyde (MDA), electrolyte leakage (EL), disparities in water and nutrient levels, chlorosis, and senescence, culminating in the death of the plant (Singh et al., 2016; Qadir et al., 2020; Muhammad et al., 2023). Heavy metals are persistent in the environment, as they are not easily removed or degraded, unlike other pollutants that can undergo degradation through chemical or biological processes. Toxic pollutants such as zinc, cadmium, copper, lead, nickel, and mercury are present in excessive amounts and pose a threat to the environment (Xie et al., 2010).

2.4 METAL TOLERANT PLANT GROWTH PROMOTING BACTERIA

Long-term heavy metal contamination in soil can function as a selection pressure to promote bacterial species able to develop heavy metal resistance (Zubair et al., 2016; Barra Caracciolo et al., 2020) which can help plants to resist stress and improve plant growth and productivity. This is possible thanks to the bacterial transformation of heavy metals into less toxic forms and the alteration of their availability (Ma et al., 2016; Tirry et al., 2018). Understanding microorganism resistance mechanisms and their relationships with plants can

make it possible to develop more efficient and specific technologies for heavy metal bioremediation to apply to crops (Jin et al., 2018). Sun et al. (2018) found that rhizosphere communities in heavy metal-contaminated soils were crop specific and specific metal–microbe interactions were found for rice, soybean or corn.

Lead (Pb) one of the most dangerous heavy metals is projected to maintain elevated levels for a period of 150 years and exhibits a soil retention span ranging from 150 to 5000 years (Yang et al., 2005). A study carried out on the utilization of *B. juncea* to mitigate the presence of heavy metals in the context of remediating soil contaminated by effluents showed that there was a significant reduction in the shoot length of *B. juncea* in Pb-contaminated soil with the increase in Pb contents. Lead contamination at 900 mg kg^{-1} significantly reduced the shoot length by up to 40% compared with the plants grown in normal soil and significant shoot fresh weight was observed. However, the application of lead-tolerant-plant-growth-promoting rhizobacteria (LTPGPR) improved the shoot length in Pb-amended soil. Although shoot dry weight of *B. juncea* was significantly reduced at all levels of lead. However, inoculating *B. juncea* with LTPGPR significantly improved the shoot dry weight in Pb-contaminated soil. A decrease in the Root length up to 52.51% at 900 mg kg^{-1} lead contamination was observed. However, inoculation of LTPGPR improved the root length up to 31.77% at the same level of contamination. The increase in Pb contents also reduced the root's fresh and dry weight. Maximum reduction of 51 and 52.74%, respectively, in root fresh and dry weight was recorded at 900 mg kg^{-1}. However, treating the plants with LTPGPR improved the root's fresh and dry weight by up to 40 and 50%, respectively (Mushtaq et al., 2023).

High levels of zinc at harmful levels in agricultural soil due to various human activities, such as the use of sewage sludge contaminated with metals or mining operations, could pose a significant threat to the production of sustainable and high-quality food (Li & Christie, 2001).

The excessive accumulation of zinc beyond its critical threshold level is detrimental to plant development as it hampers various physiological processes such as photosynthesis, enzyme functioning, and mineral uptake. Consequently, the scientific community has shown significant interest in exploring the potential of heavy metal-tolerant microorganisms for the remediation of zinc contamination (Kour et al., 2019). Key Zn-tolerant PGPR strains include the genus *Cupriavidus*, *Pseudomonas*, *Streptomyces*, *Micrococcus*, *Sphingomonas*, *Klebsiella*, *Serratia*, *Proteus* etc (Chen et al., 2014; Bhojiya and Joshi, 2016; Afzal et al., 2017; Ortiz-Ojeda et al., 2017). A study carried a pot culture experiments where plant growth-promoting effects of zinc tolerant bacteria (ZTB) isolates were investigated on maize plantlets treated with ZTB inoculants under zinc stress conditions (1,000 mg Zn/kg planting mixture). In the uninoculated control pot containing 1,000 mg Zn/kg planting mixture, there was a notable reduction in both overall plant growth and chlorophyll content as a result of Zn-induced stress when compared to control plantlets (without any Zn stress). When maize plantlets were treated with ZTB strains exhibited enhanced plant growth and chlorophyll content in comparison to the uninoculated control group (Jain et al., 2020). In a study by Yang et al. (2017) the soil properties of various Zn-contaminated sites in China were investigated. The study revealed that the levels of soil organic matter, available nitrogen, and phosphorous were significantly higher in the rhizosphere soil compared to the bulk soils at Zn-contaminated sites. This observation highlights the importance of the presence of heavy metal-tolerant plant growth-promoting rhizosphere bacteria in such environments. The bacteria's resistance to the toxic concentration of Zn is attributed to the dual mechanisms facilitated by the P-type ATPase system and the resistance-nodulation-division (RND)-driven transporters system (Spain & Alm, 2003). An investigation carried out to determine the impacts of various concentrations of (0, 100, 200, 400 and 800 mg/l) of Ni (nickel), Cd (cadmium), Pb (lead), Cr (chromium) and Hg (mercury) on seed germination and seedling growth of *Sorghum bicolor* showed that upon a comprehensive

assessment of the interactions, it was observed that the 400 and 800 mg/l does of Cd, Ni and Hg significantly decreased root fresh weight. The influence of Pb and Cr on root fresh weight was more constrained than that of other heavy metals (Ertekin et al., 2020).

2.5 IRON-CHELATING MOLECULES: THE ROLE OF SIDEROPHORES IN PLANT GROWTH ENHANCEMENT FOR HEAVY METAL AFFECTED LANDS.

Numerous studies have indicated that plant growth-promoting rhizobacteria (PGPR) could offer a promising solution for alleviating the negative impact of deteriorated lands, particularly those affected by heavy metal conditions (Bhojiya et al., 2022). The symbiotic relationship between the rhizosphere and microbes plays a pivotal role in iron-stressed degraded areas due to the production of iron-chelating molecules like siderophore (Dertz et al., 2006). Siderophore-producing microbes provide plants with Fe nutrition to enhance their growth when the bioavailability of Fe is low (Crowley, 2006).

The term siderophore originates from the Greek words sidero, signifying “iron” and phore indicating “carriers” referring to iron-bearing compounds that uptake insoluble iron from various environmental origins (Nagoba and Vedpathak, 2011). Siderophores are categorized based on their functional groups, namely hydroxamate-type siderophore (i.e. ferrioxamine B, catecholate-type siderophore (i.e. enterobactin), carboxylate-type siderophore s (i.e. rhizobactin) and mixed ligand siderophore (i.e. pyoverdine) (Ito and Butler, 2005; Zawadzka et al., 2006; Butler and Theisen, 2010). Some bacterial species, like *P. aeruginosa*, can produce pyoverdin hydroxamate type of siderophore under limited iron conditions (Meneely and Lamb, 2007). Some common bacterial species such as *Escherichia coli, Salmonella typhimurium, and Klebsiella pnemoniae* dominantly produce enterochelin subtypes of catecholate types of siderophore (Dertz et al., 2006). The molecular mass of microbial siderophores, ranges between 200 and 2000 Da, while phytosiderophores produced by plants range between 500 and 1000 Da (Neilands, 1981).

In iron-stressed degraded soil, plants secrete siderophore from their roots to regulate the iron levels necessary for their metabolic and physiological functions, albeit not always achieving optimal levels (Herlihy et al., 2020). Several researchers have demonstrated that siderophore-producing rhizobacteria that promote plant growth were demonstrated, for example, *Bacillus subtilis*, *B. licheniformis, B. coagulanse, B. circulance, Pseudomonas koreensis, P. fluroscence* (Ghazy and El-Nahrawy, 2021), *P. aeruginosa* (Subramanium and Sundaram, 2020; Singh et al., 2021a), *Pseudoalteromonas tetraodonis, Bacillus cereus, Psychrobacter pocilloporae, Micrococcus, aloeverae, Pseudomonas weihenstephanensis* (Sinha et al., 2019), *Pseudomonas sp.* (Singh et al., 2022), *Enterobacter genera*, *Bacillus*, and *Rhodococcus* (Sah and Singh, 2015), *Bacillus megaterium* (Singh et al., 2020a), *Pantoae cypripedii* (Singh et al., 2021b), *Kosakonia radicincitans* (Singh et al., 2020b), and *Pantoae dispersa* (Singh et al., 2021c).

Siderophore-producing microbes (SPM) generate various iron-chelating compounds, which help alleviate plant stress in iron-stressed soil serve as a signature for sustainable agriculture and prove environmentally friendly for crop cultivation in degraded lands (Alam, 2014). Siderophores play a crucial role in several functions of plants such as respiration (Aznar and Dellagi, 2015), photosynthesis (Nagata et al., 2013), bioremediation (Saha et al., 2016), plant growth promotion (Yadav et al., 2011; Ghazy and El-Nahrawy, 2021), and phytoremediation of heavy metals (Kong and Glick, 2017; Leguizamo et al., 2017; Ustiatik et al., 2021).

Siderophores are also capable of altering the oxidation states of heavy metals such as Cd, Cu, Ni, Pb, Zn, Th, U, and Pu, rendering them less harmful (Schalk et al., 2011). Siderophores can bind to different toxic metals such as Cr^{3+}, $Cu^{3+,}$ Pb^{2+}, Cu^{2+}, V^{4+}, and Al^{3+}, and thus toxic heavy metals do not hinder the efficiency of plant cells. However, the siderophore's binding capability to Fe is higher than toxic heavy metals (Baysse et al., 2000;

Braud et al., 2009b). Furthermore, a variety of metals, i.e. Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Eu^{3+}, Ga^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Tb^{3+}, Tl^{+} and Zn^{2+}, could be chelated by the siderophore pyochelin produced by *Pseudomonas aeruginosa.* Nevertheless, the uptake process did not seem to assimilate any metal other than Fe^{3+} (Braud et al., 2009a). Thus, the toxic heavy metal detoxifying and binding ability of siderophore plays a remarkable role in plant growth under heavy metal-polluted soil, therefore siderophores become a useful tool in bioremediation, which is a cost-effective and environmentally friendly technique (Rajkumar et al., 2010). With a high affinity for iron-chelating compounds, siderophore initiates a bioremediation process that enhances nutrient absorption, promotes plant growth, and reduces heavy metal toxicity (Rajkumar et al., 2010). Siderophore-producing *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* are known to increase the rate of phytoextraction and phytoremediation of heavy metals (Braud et al., 2009a).

2.6 BIOREMEDIATION: AN ECO-FRIENDLY APPROACH TO CONTAMINATED SITES

Remediation of polluted areas through traditional approaches like landfilling, soil washing, electrokinetic remediation, and excavation is characterized by high costs and substantial energy consumption (Jeyasingh & Philip, 2005). In this context, an alternative strategy known as “Bioremediation” has gained increased attention for the remediation of contaminated sites owing to its cost-efficient and environmentally friendly characteristics (Wu et al., 2006). To achieve effective remediation, microorganisms inhabiting metal-polluted environments need to adapt to the presence of heavy metal stress (Ma et al., 2011). The bacterial strains exhibit the ability to withstand heavy metal stress through various resistance mechanisms, such as forming complexes with thiol-containing molecules, employing active efflux systems, immobilizing/mobilizing heavy metals, sequestering them extra or intracellularly, and transforming highly toxic compounds into less harmful forms (Bruins et al., 2000; Gibbons et al., 2011).

Certain bacterial strains, like Pseudomonas fluorescence, produce pyoverdines siderophore, which enhances mobility and diminishes the toxicity of heavy metals in uranium mining areas (Edberg et al., 2010). *Streptomyces tendae* F4 is known to reduce the translocation of cadmium (Cd) from the rhizosphere to plants in heavy metal-polluted soil (Dimkpa et al., 2009). A study reported that *Providencia sp.* (TCR05) and *Proteus mirabilis* (TCR20) showed reduced Cr toxicity from Cr(VI) to Cr(III) and enhanced plant pigments, protein, phenolics, and relative water content, while proline, lipid peroxidation, and superoxide dismutase decreased in *Zea mays* under heavy metal contaminated and drought conditions (Vishnupradeep et al., 2022). *Agrobacterium radiobacter* produces siderophores which removed approximately 54% of the As from a metal-contaminated soil (Wang et al., 2011). Recently, pyoverdines have been shown to mobilize U(VI), Np(V) and other metals from uranium mine waste (Behrends et al., 2012). Not only do microbial siderophores play a role in metal bioremediation, but many studies have also demonstrated that phytosiderophores are efficient in mobilizing metals in soil (Rajkumar et al., 2009; 2010; 2012). According to Ruggiero et al. (1999), phytosiderophores exhibit a high affinity for complexation with several metals in the following order ($Cd^{2+} > Ni^{2+} > Pb^{2+} > Sn^{2+} > AsO^{4-2} > AsO^{2-1} > Mn^{2+} > Co^{2+} > Cu^{2+} > Fe^{+3}$) and very weakly binds Al^{3+} and Cr^{3+}.

Synergistic collaborations between plants and metal-resistant plant growth-promoting rhizospheric bacteria offer significant potential for the remediation of contaminated soils (Babu et al., 2015). The PGP bacteria play a pivotal role in mitigating metal toxicity and thereby exerting beneficial effects on plant growth and mineral uptake (Gururani et al., 2013). These rhizospheric bacteria promote plant growth through mineral phosphate solubilization, nitrogen fixation, indole-3-acetic acid, siderophores, hydrogen cyanide, and ammonia production (Khan et al., 2009). These microbes inhabiting the plant rhizosphere are essential to the phytoremediation process due to their enhanced biomass production, plant growth, anti-oxidative enzymes, and metal tolerance ability. Symbiotic association between plant and SPM

may play a role in heavy metal uptake, SPM *Rhizobium* strains enhanced Cu uptake while the *Pseudomonas* strain promoted Cu and Fe uptake by *Phaseolus vulgaris* plants (Carrillo-Castaneda et al., 2007). While *S. acidiscabies* SPM secretes hydroxamate types of siderophores responsible for the solubilization and uptake of nickel and iron by *Vigna unguiculata* plants under nickel stress conditions (Dimkpa et al., 2008). The symbiotic association of SPM *Kluyvera ascorbata* and plants decreased the toxicity of heavy metals (Burd et al., 2000). By applying selected rhizospheric bacteria it is possible to enhance metal phytoavailability while reducing toxicity, resulting in increased biomass and accumulation of significant amounts of metals (Ma et al., 2011).

CHAPTER 3: METHODOLOGY

3.1 COLLECTION OF SOIL SAMPLE

The rhizosphere soil sample was collected from the *Jaya* rice variety growing near mining sites at Bicholim Taluka in the North Goa district of Goa. An intact root system was dug out and the rhizosphere soil samples were carefully taken in polythene bags and stored at 4˚C in the refrigerator for further studies.

3.2 ISOLATION OF RHIZOSPHERE-INHIBITED BACTERIA

The isolation of rhizospheric bacteria was carried out by plating serial dilutions of the soil sample on nutrient agar plates. The Nutrient Agar composition (g L^{-1}): Peptone 5g, Sodium chloride 5g, Beef extract 1.5g, Yeast extract 1.5g and Agar 15g. Nutrient agar media was prepared by suspending all the ingredients in 1 L distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Aseptically poured into sterile Petri plates.

Roots were washed with distilled water to remove non-rhizosphere soil. Roots were cut from the stem portion and rinsed in sterile distilled water in laminar airflow. Using sterilised scissors roots were cut into pieces and placed in a sterile glass vial containing 2 ml of sterile distilled water. The vial was capped and shaken vigorously for 5 minutes. This soil suspension was serially diluted to 10^{-5} and 100 µL from each dilution was homogeneously spread plated on Nutrient agar plates. Plates were incubated at 30˚C for 24-48 hrs. The colonies were distinguished, sub-cultured, and purified for further analysis.

3.3 SCREENING OF SIDEROPHORE-PRODUCING BACTERIA

3.3.1 Qualitative Screening of Siderophore-Producing Bacteria

The Chrome Azurol Sulfonate assay (CAS) agar plate method was used for qualitative screening of siderophore production of purified bacterial isolates (Schwyn and Neilands, 1987).

Chrome Azurol Sulphonate (CAS) solution: Solution A: Dissolved 0.06 g of CAS in 50 mL of deionized water; Solution B: 0.0027g $FeCl_3 \cdot 6H_2O$ dissolved in10 mL 10mM HCl; Solution C: 0.073 g of CTAB (Cetyl trimethylammonium bromide) in 40 mL of deionized water. Solution A was slowly added to solution B along the wall of the beaker, and subsequently, solution C was added to the ferric-CAS solution while stirring, stabilizing the CAS-Fe complex to form a blue-coloured solution. Nutrient agar and CAS solutions were sterilized at 121 °C for 15 min separately. The prepared blue CAS solution was added to the medium at a ratio of 10:100 to make CAS-agar plates. All the bacteria isolated were spot inoculated on CAS-agar plates and incubated at 30 °C for 7 days.

On CAS blue agar, an orange halo formed around colonies indicates the ability of the bacterial strains to produce siderophore by consuming the iron present in the medium. The size of the halo diameter/colony diameter ratios was screened and the results are shown as the mean ± standard deviation (SD).

3.3.2 Detection of Siderophore Chemical Nature by Ferric Chloride ($FeCl_3$) Test

Catecholate and hydroxamate-type siderophores were differentiated by the $FeCl_3$ test (Neilands, 1981). The bacterial isolates were grown in a nutrient broth medium at 30˚C for 24-48 hrs on a rotary shaker (120 rpm) and then centrifuged for 15 min at 3000 rpm. 1 mL of culture supernatant was added to 1 mL of 2% $FeCl_3$ solution. Measure the OD of the supernatant at 450 nm and 495 nm. In this test, the formation of wine-coloured ferric catecholate with λ_{max} at 495nm indicates the presence of catecholate siderophore. The formation of orange coloured ferric hydroxamate, showing λ_{max} ranging from 450nm indicates hydroxamate siderophore (Neilands, 1981).

3.4 COLONY MORPHOLOGY AND GRAM STAINING

The morphological characteristics of bacterial colonies were observed and recorded using distinctive features such as shape, colour, elevation, opacity, margin, surface and texture of the colony. The gram character of the bacterial isolates was studied using Gram staining. A thin smear of bacterial culture was prepared on a clear, dry glass slide. It was allowed to air dry and was fixed by gentle heat. The smear was flooded with Gram's Crystal Violet for 1 minute. The slide was washed with tap water. Next, the smear was flooded with Gram's Iodine and allowed to remain for 1 minute. Gram's Decolorizer was used to decolourize the smear until the blue dye no longer flowed from it. Another wash with tap water followed. The smear was counter-stained with 0.5% w/v Safranin for 20 seconds and rinsed off with water. Finally, the slide was allowed to air dry and examined under an oil immersion objective. Gram staining is a fundamental technique for distinguishing between Gram-positive and Gram-negative bacteria based on differences in cell wall composition where Gram-positive bacteria appear violet coloured while Gram-negative appear pinkish red coloured.

3.5 TOLERANCE OF SIDEROPHORE-PRODUCING BACTERIA TOWARDS DIFFERENT METALS: LEAD AND ZINC.

Lead (Pb) and Zinc (Zn) were used as two metal treatments. The metal salts used for the study include lead acetate $Pb(C_2H_3O_2)_2$ and Zinc sulphate heptahydrate $ZnSO_4 \cdot 7H_2O$. Stock solutions of these metals were prepared by adding 1.569g and 4.397g in 10 mL distilled water respectively. These metal stock solutions were sterilized by filtering through membrane filters of pore size 0.22 µm and added to the nutrient agar medium after autoclaving and cooling to 45 to 50°C to get a range of concentrations of 200, 250, 300, 350, 400, 450 and 500 mg/L media by adding 1, 2, 3, 4 and 5 mL of the Pb and Zn stock solution in 1 L media. Nutrient agar without metal was used as a control. Isolated siderophore-producing bacterial strains were inoculated on each of these treatment media plates. Metal resistance was determined by the

growth of bacterial colony after 7 days of incubation at 30°C. The minimum inhibitory concentration (MIC) of Pb and Zn at which no colony growth occurred was determined. The lowest concentration of Pb and Zn that inhibited the growth of siderophore-producing bacteria was taken as the MIC of that metal.

3.6 DETERMINATION OF PHOSPHATE-SOLUBILIZATION

The phosphate solubilizing ability of bacterial isolates was analyzed on a Pikovskaya agar medium. The Pikovskayas Agar composition (g L^{-1}): Yeast extract 0.5g, Dextrose 10g, Calcium phosphate 5g, Ammonium sulphate 0.5g, Potassium chloride 0.2g, Magnesium sulphate 0.1g, Manganese sulphate 0.0001g, Ferrous sulphate 0.0001g and Agar 15g. Pikovskaya agar media was prepared by suspending all the ingredients in 1 L distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and aseptically poured into sterile Petri plates.

The bacterial isolates were spot inoculated on Pikovskaya agar plates and incubated at 30˚C for 7 days. A clear zone around colonies indicated the phosphate-solubilizing ability of isolates. The colony diameter and halo zone diameter were measured in cm and the solubilization index (SI) was calculated using the following formula:

$$SI=(CD+HD)/CD$$

where CD is the colony diameter, and HD is the halo zone diameter.

3.7 QUALITATIVE ANALYSIS OF NITROGEN-FIXING ABILITY

The nitrogen-fixing ability of the bacterial isolates was evaluated based on their ability to grow on N-free Jensen's media. The Jensen's media composition (g L^{-1}): Sucrose 20g, Dipotassium hydrogen phosphate 1g, Magnesium sulphate 0.5g, Sodium chloride 0.5g, Ferrous sulphate 0.1g, Sodium molybdate 0.005g, Calcium carbonate 2g and Agar 15g. The Jensen

media was prepared by suspending all the ingredients in 1 L distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes, aseptically poured into sterile Petri plates. The bacterial isolates were spot inoculated on Jensen media plates and incubated at 30°C for 48 hrs (Jimtha et al., 2014; Kumar S. et al., 2018). The bacterial colony which can grow on this media is considered nitrogen-fixing.

3.8 PLANT GROWTH-PROMOTING TRAITS OF SIDEROPHORE-PRODUCING BACTERIA AT HIGH CONCENTRATIONS OF: LEAD AND ZINC

3.8.1 Siderophore-Producing Ability at High Concentration of Lead and Zinc

Qualitative Estimation of Siderophore Production in the presence of a high concentration of metals using Chrome Azurol Sulfonate assay (CAS) agar plates which were prepared as mentioned previously with the addition of 300mg Pb/L and 200mg Zn/L respectively. Bacterial isolates were spot inoculated on these CAS-agar plates and incubated at 30 °C for 7 days. The colony diameter and halo zone diameter were measured in cm. The size of the halo diameter/colony diameter ratios was screened and the results are shown as the mean ± standard deviation (SD).

3.8.2 Phosphate-Solubilizing Ability at High Concentration of Lead and Zinc

Pikovaskaya agar media was prepared as mentioned previously but by adding 300mg Pb/L and 200mg Zn/L respectively. The bacterial isolates were spot inoculated on these Pikovskaya agar plates and incubated at 30˚C for 7 days. The colony diameter and halo zone diameter were measured in cm and the solubilization index (SI) was calculated as mentioned previously.

3.8.3 Nitrogen-Fixing Ability at High Concentration of Lead and Zinc

The Jensen media was prepared as mentioned previously but by adding 300mg Pb/L and 200mg Zn/L respectively. The bacterial isolates were spot inoculated on these Jensen media

plates and incubated at 30°C for 48 hrs. The bacterial colony which can grow on this media is considered to be nitrogen-fixing.

3.9 TOLERANCE OF SIDEROPHORE-PRODUCING BACTERIA TOWARDS DIFFERENT ANTIBIOTICS

Ampicillin, kanamycin, and streptomycin were used as three different antibiotic treatments. Stock solutions of these antibiotics were prepared by dissolving 1g of each of these antibiotics in 10 mL of distilled water respectively. These stock solutions were sterilized by filtering through membrane filters of pore size 0.22 µm and 1 mL of each of these antibiotic stock solutions were added to 1 Liter nutrient agar medium after autoclaving and cooling to 45 to 50 °C. The final concentration of each of these antibiotics was 100 µg/mL medium. A nutrient agar medium without antibiotics was used as a control. Isolated siderophore-producing bacterial strains were inoculated on each of these treatment media plates. Antibiotic resistance was determined by the growth of the bacterial colony after 3 to 4 days of incubation at 37°C.

3.10 DETERMINATION OF PLANT GROWTH PROMOTION BY THE SELECTED BACTERIA IN THE PRESENCE OF LEAD AND ZINC.

The pot experiment for selected siderophore-producing isolates showing high MIC values was conducted. Seeds of rice variety IR64 used in this experiment were obtained from Zonal Agricultural Research Station, V C Farm, Mandya, Karnataka. The soil for this experiment was collected from Ashish Agro Associates, St. Inez, Panjim, Goa. The soil was sieved and sterilized in a ratio of 3:1 (Soil: Compost). The soil was contaminated using lead acetate $Pb(C_2H_3O_2)_2$ and zinc sulphate heptahydrate $ZnSO_4 \cdot 7H_2O$ as a source of Pb^{2+} (300 mg/kg) and Zn^{2+} (200 mg/kg) respectively and kept for one week to stabilize.

Seeds were surface sterilized using 70% ethanol for 30 sec followed by 2% sodium hypochlorite solution for 1 minute and washed three times with sterile distilled water. These seeds were soaked for 2 days in sterile distilled water after which they were transferred to pots

with and without Pb^{2+} and Zn^{2+} amendment. A total of 9 pots were used per treatment including a control and 25 seeds were sown in each pot in a growth room under controlled conditions at 28°C and 70% relative humidity.

The inoculum for the pot experiment was prepared in nutrient broth at 28±2°C and was added to the pots having cfu $10^8 – 10^9$ ml^{-1}. On the seventh day, the seedlings were treated with bacterial isolates designated as B1 and B2 respectively. The pots were irrigated with distilled water on an alternative day until the completion of the study. The details of the treatments: C (control, uncontaminated soil and no bacterial treatment), B1 (uncontaminated soil, treated with B1 strain), B2 (uncontaminated soil, treated with B2 strain), Pb (Pb^{2+} contaminated soil with 300 mg Pb/kg), B1+Pb (Pb^{2+} contaminated soil with 300 mg Pb/kg, treated with B1 strain), B2+Pb (Pb^{2+} contaminated soil with 300 mg Pb/kg, treated with B2 strain), Zn (Zn^{2+} contaminated soil with 200 mg Zn/kg), B1+Zn (Zn^{2+} contaminated soil with 200 mg Zn/kg, treated with B1 strain) and B2+Zn (Zn^{2+} contaminated soil with 200 mg Zn/kg, treated with B2 strain). Each treatment had three replicates.

Whole plants were harvested on the 7th day of sowing before bacterial treatment and on the 7th and 14th day after bacterial treatment. Root and shoot lengths (cm) were measured of the plants on the day of harvest. Fresh weight and dry weight (g $plant^{-1}$), were noted after drying for 3 days at 70°C. Water content and water content percentage were calculated using the following formula:

$$\text{Water content} = \text{Fresh weight} – \text{Dry weight}$$

$$\text{Water content \%} = (\text{Water content}/\text{Fresh Weight}) \times 100$$

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Isolation of Rhizosphere-Inhibited Bacteria

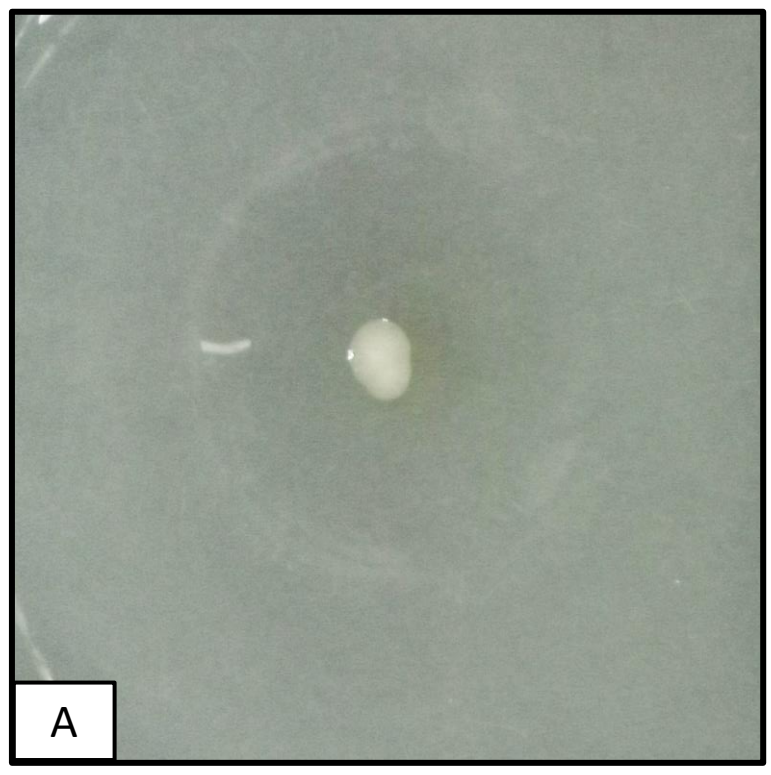
In the present research, the rhizosphere soil of the *Jaya* rice variety was serially diluted and a total of 17 morphological different bacterial colonies were recovered, purified and preserved for further experiments. Only a sterile water-streaked plate with media was maintained as control.

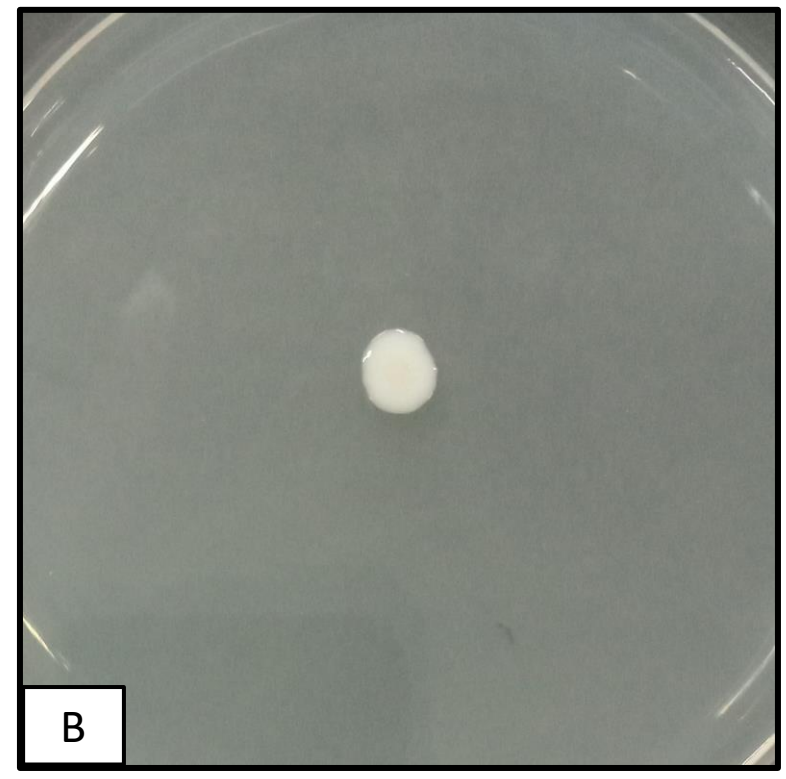
4.2 Qualitative Screening for Siderophore-Producing Bacteria

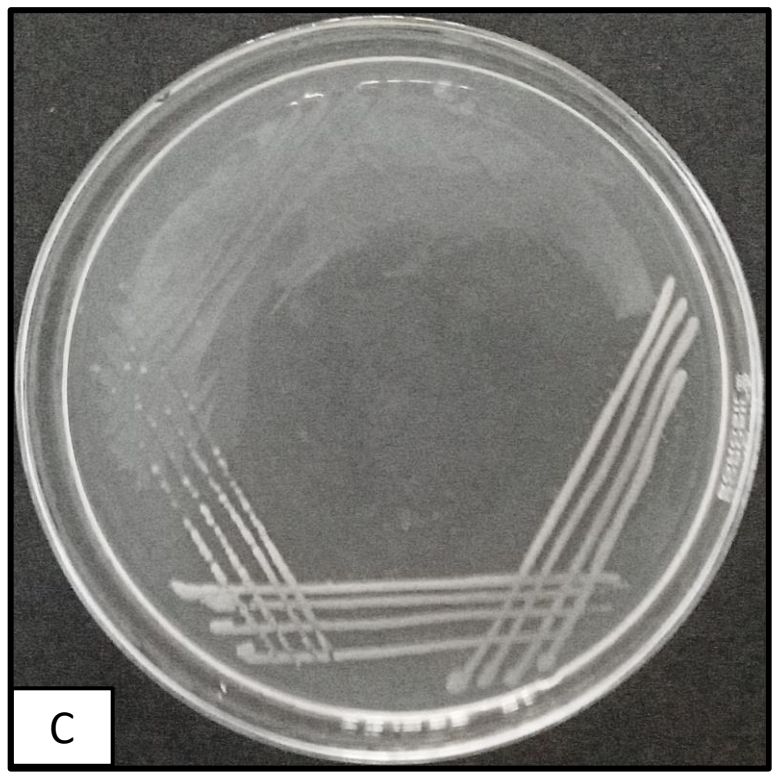
According to the CAS-agar assay, 6 out of 17 bacterial isolates exhibited an orange-yellow halo after 7 days of incubation at 30˚C on the CAS-agar plate and therefore were considered positive for siderophore production. The change in the blue colour of CAS-agar is in response to iron chelation by bacterial isolates. Among the strains, the intensity of the orange-yellow halo and diameter showed wide variation which ranged from 2.6 cm to 0.4 cm after 7 days of incubation. The colonies that exhibited orange zones were inoculated using the streak plate method on a fresh CAS agar plate. Each single colony was obtained by repeating the process several times. The colony diameter and halo zone diameter were measured in cm in triplicates for each bacterial isolate. Out of these 6 isolates, 2 bacterial strains that showed maximum halo with diameter above 2 cm were selected and designated as B1 and B2 (Figure 4.1 A, B, C and D) and were selected for further analysis. The size of the halo diameter/colony diameter ratios was screened and the results are shown as the mean ± standard deviation (SD) The average ratio of B2 was 2.59 higher than that of B1 with an average ratio of 1.97 (Table 4.1) (Figure 4.2 A and B).

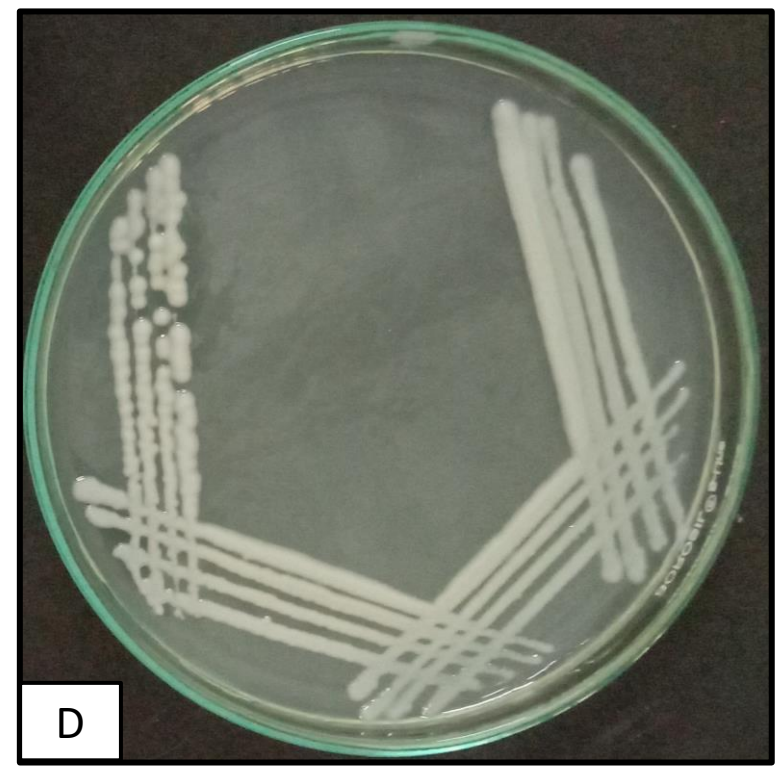
4.3 Detection of Siderophore Chemical Nature by Ferric Chloride ($FeCl_3$) Test

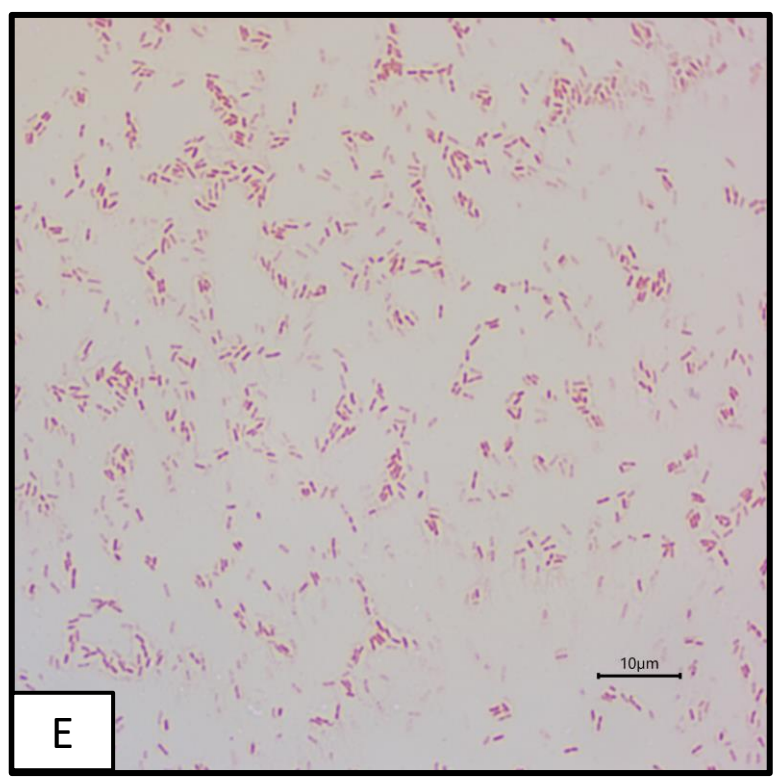
In the $FeCl_3$ test, the spectral analyses of siderophores showed maximum absorbance of 495, which confirms the catecholate nature of the siderophores. Both the bacterial isolates











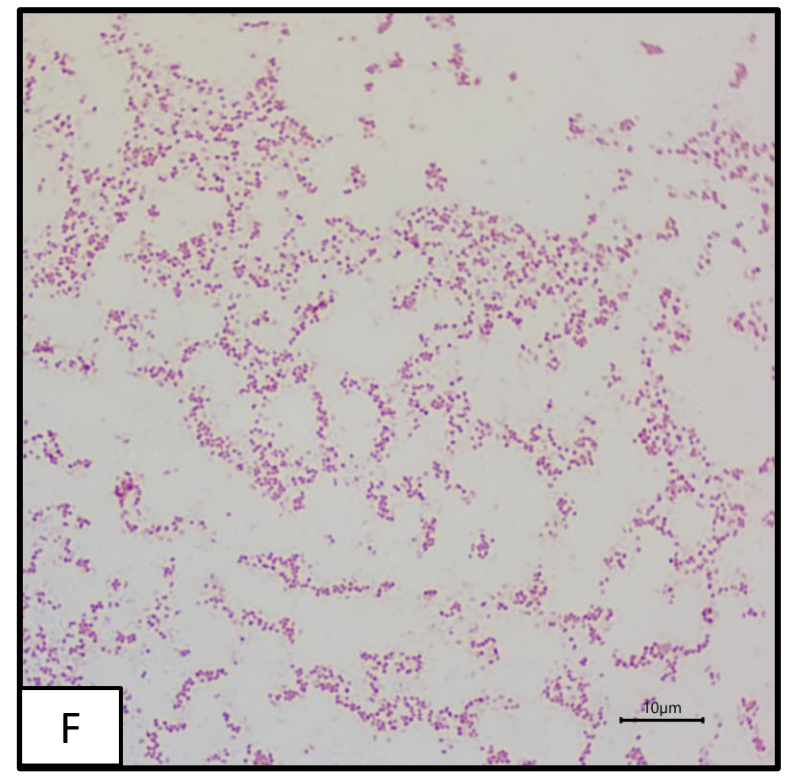
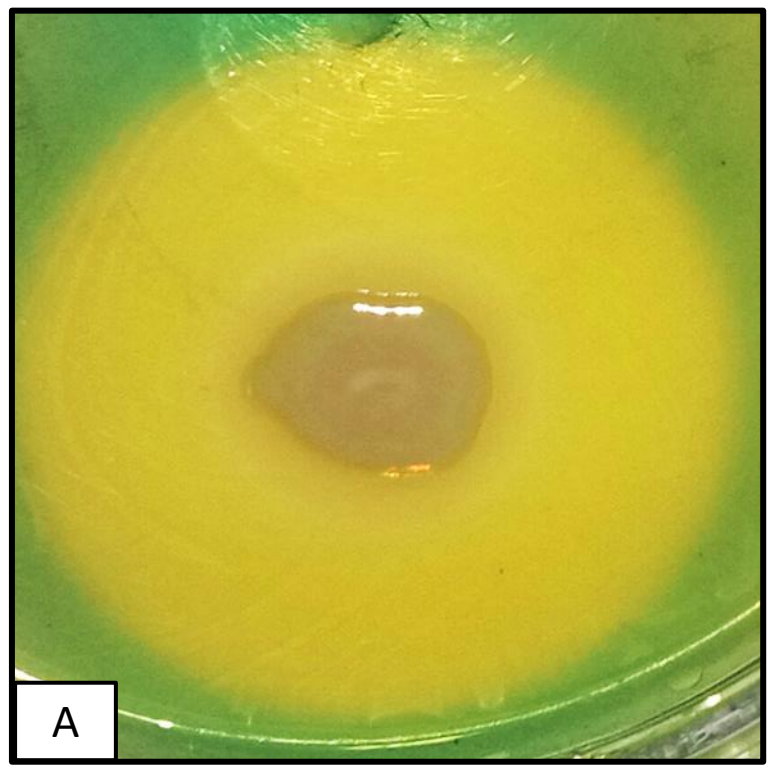
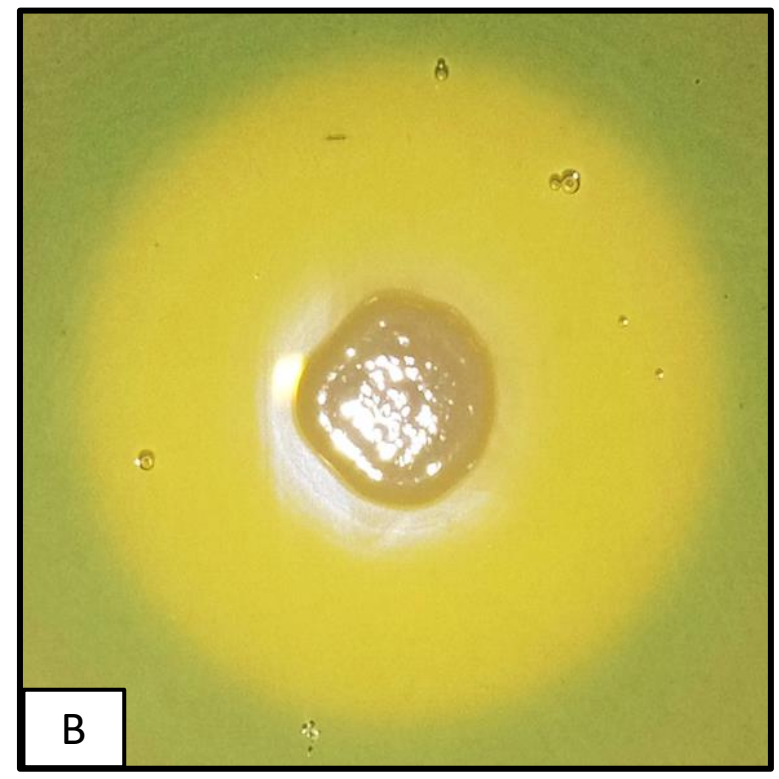
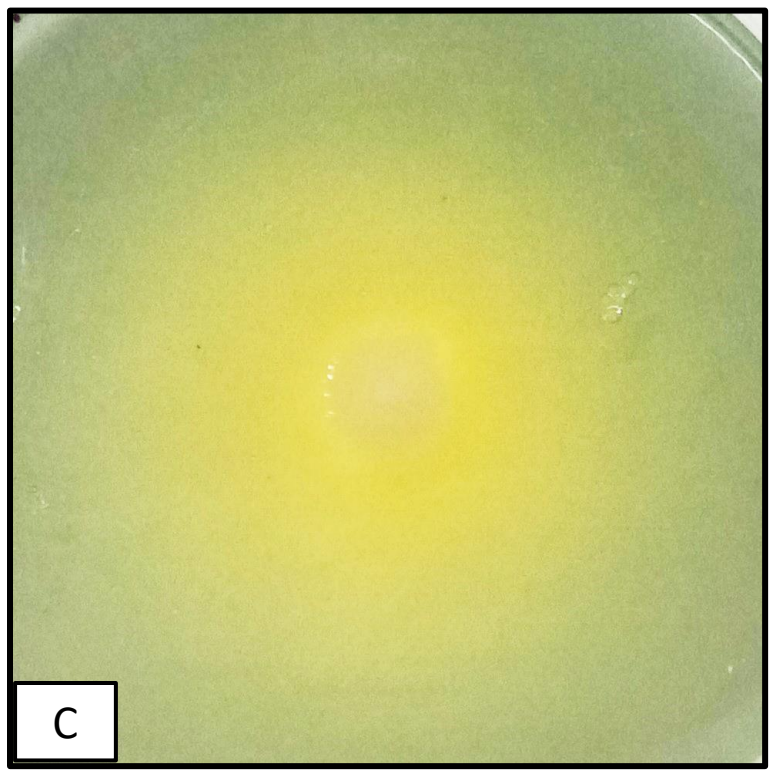
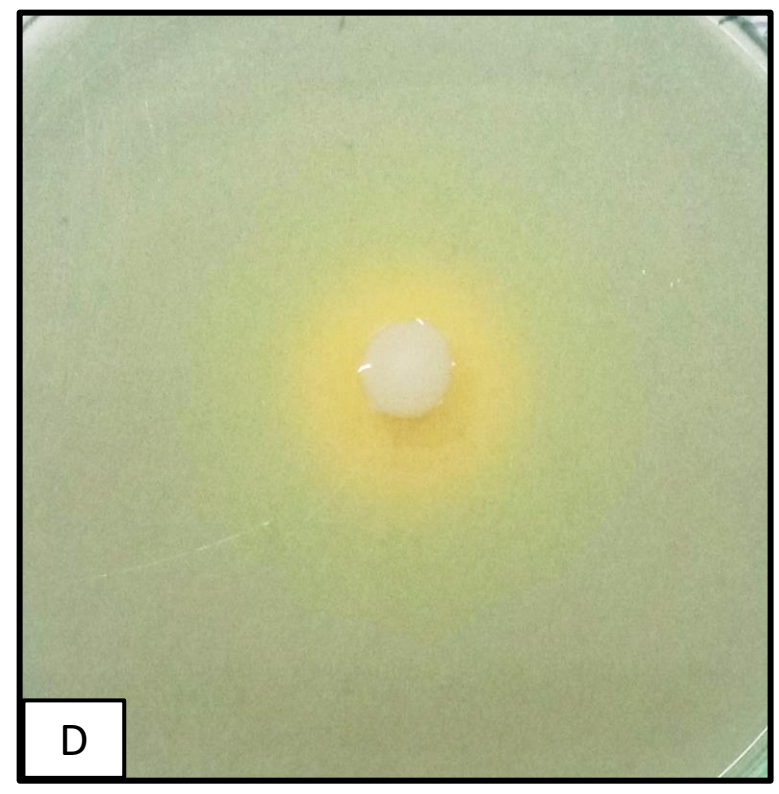


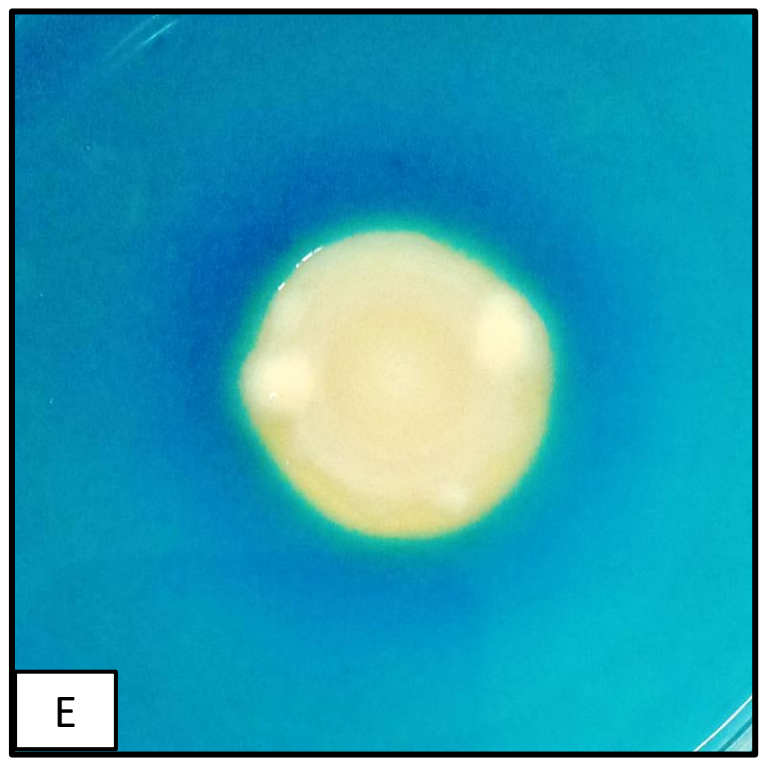
Figure 4.1 Isolated B1 and B2 bacteria from rhizosphere soil. (A) B1 spot inoculated on the nutrient agar plate, (B) B2 spot inoculated on the nutrient agar plate, (C) B1 quadrant streak on the nutrient agar plate, (D) B2 quadrant streak on nutrient agar plate, (E) B1 gram-negative and (F) B2 gram-positive.











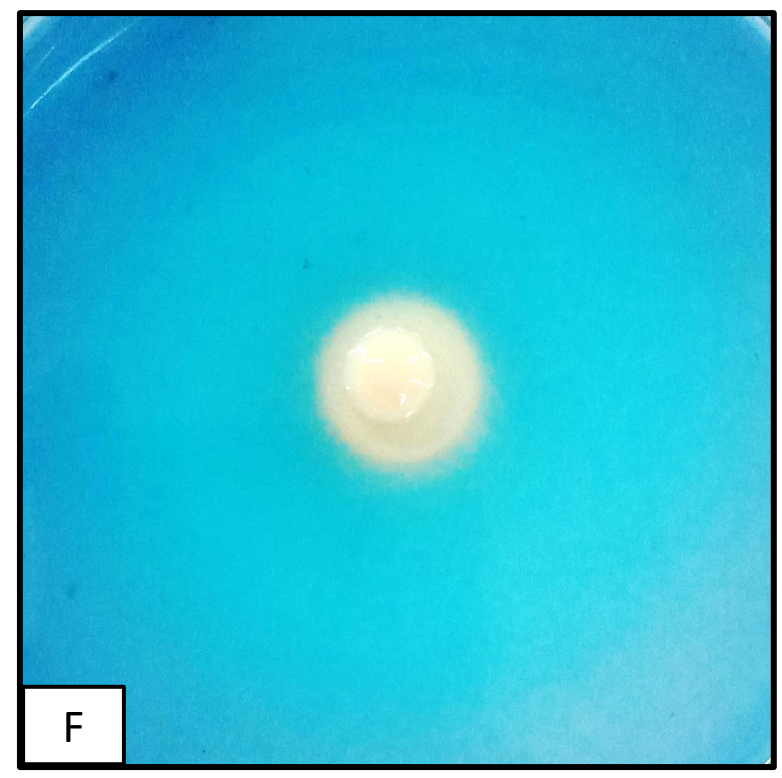


Figure 4.2 Siderophore production on CAS-agar plates is indicated by a halo around the bacterial colony. (A) B1 on the CSA-agar plate, (B) B2 on the CAS-agar plate, (C) B1 on the CAS-agar plate containing Pb, (D) B2 on the CAS-agar plate containing Pb, (E) B1 on the CAS-agar plate containing Zn, (F) B2 on the CAS-agar plate containing Zn.

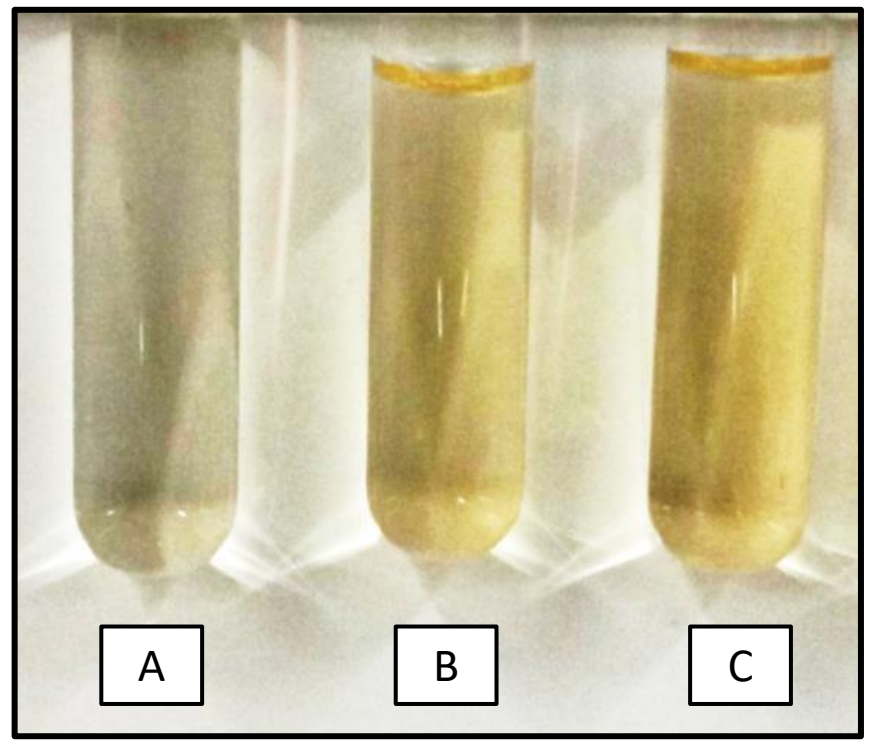


Figure 4.3 Ferric Chloride ($FeCl_3$) test for the detection of the type of siderophore. (A) Blank, (B) B1 and (C) B2.

Table 4.1 Qualitative estimation of siderophore production and phosphate solubilization.

ISOLATES	**RATIO OF HALO DIAMETER/COLONY DIAMETER**			**PHOSPHATE SOLUBILIZATION INDEX (SI)**		
	CAS-agar	CAS-agar		Pikovskaya-agar	Pikovskaya-agar	
		Pb	Zn		Pb	Zn
B1	1.97±1.11	1.33±0.11	1.24±0.14	2.30±0.02	1±0	-
B2	2.59±0.76	1.84±0.04	1.54±0.29	3.08±0.19	1±0	2.27±0.12

The values are the means of three replicates with standard deviation (±SD).

Table 4.2 Morphological characteristics and gram character of bacterial isolate.

ISOLATES	**B1**	**B2**
Shape	Rod	Coccus
Form	Circular	Circular
Colony colour	White	White
Surface	Shiny and smooth	Shiny and smooth
Elevation	Convex	Convex
Opacity	Translucent	Opaque
Margin	Entire	Entire
Texture	Butyrous	Butyrous
Gram character	Gram-negative	Gram-positive

B1 and B2 showed the catecholate nature of the siderophore by forming a yellow colour (wine colour) (Figure 4.3).

4.4 Morphological Characterization and Gram Staining

The colony morphology of the pure bacterial isolates was examined. Gram staining was done as per the universal standard method. (Figure 4.1 E and F) (Table 4.2)

4.5 Tolerance of Siderophore-Producing Bacteria Towards Lead and Zinc

Both the bacterial isolates B1 and B2 were subjected to metal stress to determine their minimum inhibitory concentration which represents the lowest concentration of metal at which bacterial growth was inhibited. These bacterial strains were exposed to varying concentrations of lead (Pb) and zinc (Zn): 200, 250, 300, 350, 400, 450, and 500 mg/L medium. B1 strain exhibited higher tolerance to Pb (MIC = 400 mg/L) than to Zn (MIC = 250 mg/L). B2 strain showed similar behaviour, with a higher MIC for Pb (350 mg/L) compared to Zn (300 mg/L).

From these MIC values the concentration of Pb and Zn was selected as 300 mg/L and 200 mg/L which fall within the range where both B1 and B2 strains exhibit their respective MICs to explore bacterial responses to heavy metal stress while ensuring consistency across both strains (Figure 4.4).

4.6 Determination of Phosphate Solubilization

The bacterial isolates were screened for phosphate solubilization on the Pikovskaya medium and both B1 and B2 showed the development of a sharp phosphate solubilization zone after 7 days of incubation. The colony diameter and halo zone diameter were measured in cm in triplicates for each bacterial isolate and the Solubilization Index was calculated (Table 4.1). B2 showed a higher phosphate solubilization index i.e. 3.08 than B1 with 2.3 (Figure 4.5 A and B).

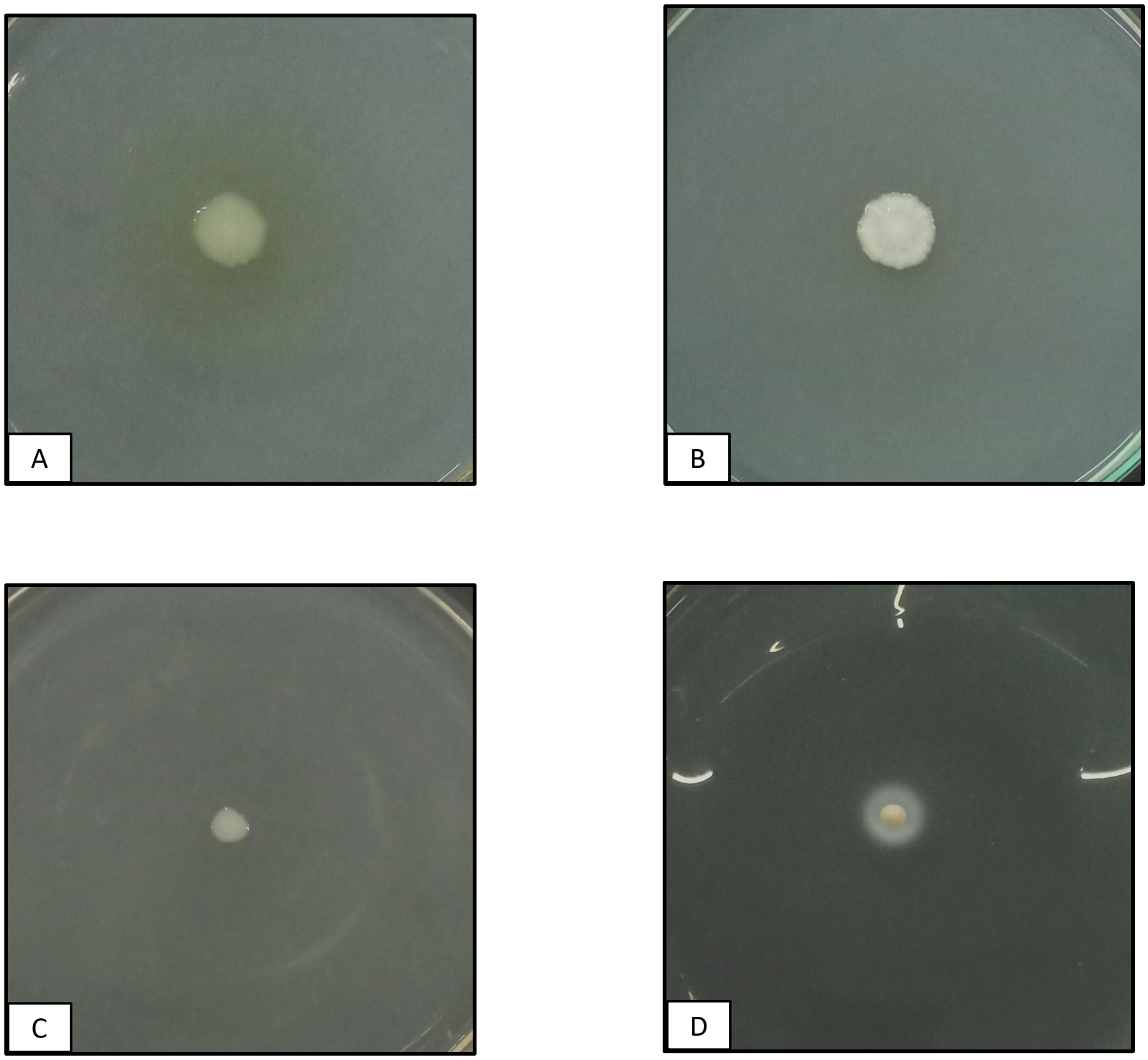


Figure 4.4 Tolerance of siderophore-producing bacteria to different metals represented by bacterial growth on metal-containing medium. (A) B1 on the Nutrient agar plate containing Pb, (B) B2 on the Nutrient agar plate containing Pb, (C) B1 on the Nutrient agar plate containing Zn and (D) B2 on the Nutrient agar plate containing Zn.

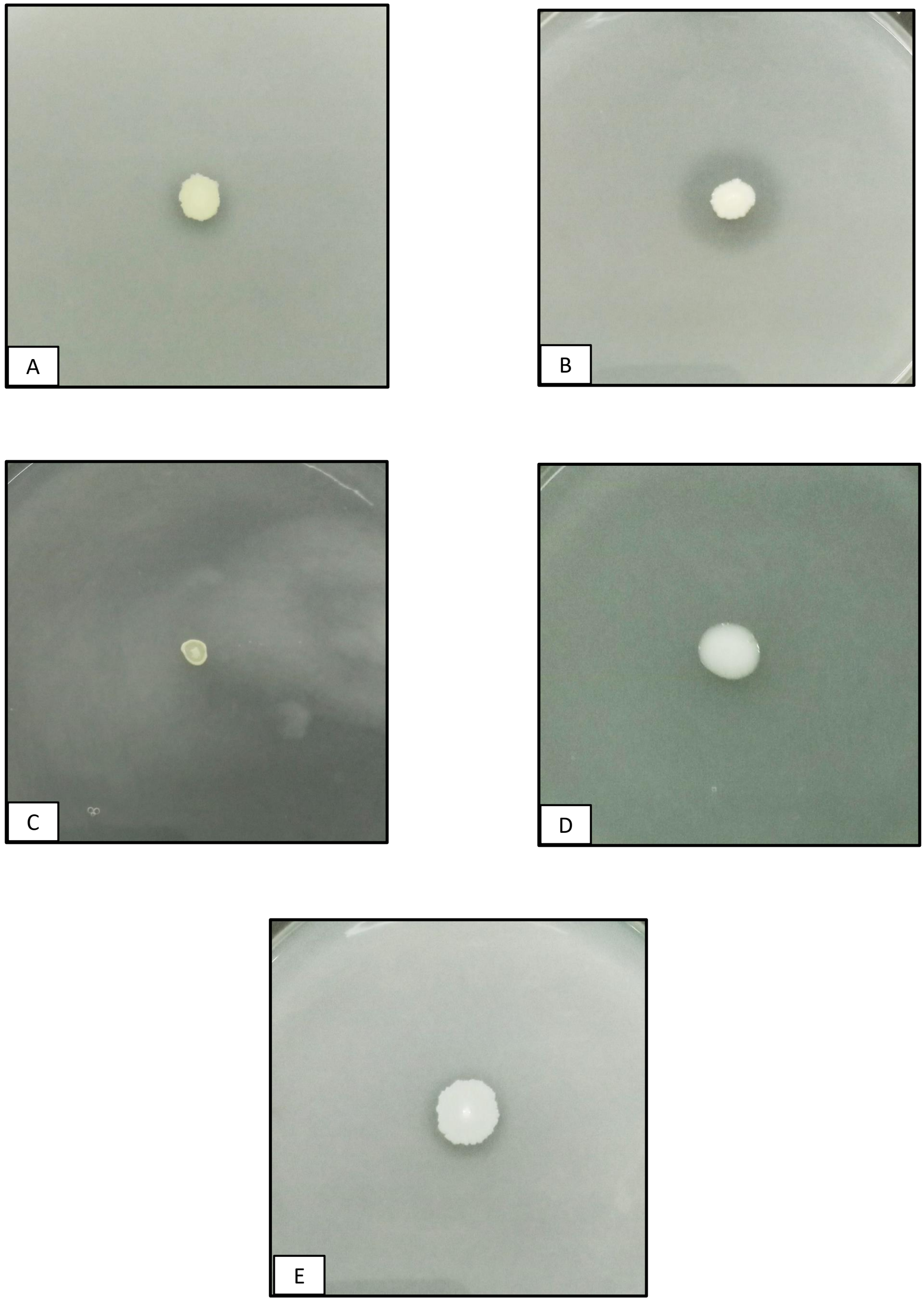


Figure 4.5 Phosphate solubilization on Pikovskaya plates is indicated by a clear zone around the bacterial colony. (A) B1 on the Pikovskaya plate, (B) B2 on the Pikovskaya plate, (C) B1 on the Pikovskaya plate containing Pb, (D) B2 on the Pikovskaya plate containing Pb and (E) B2 on the Pikovskaya plate containing Zn.

4.7 Determination of Nitrogen-Fixing Ability

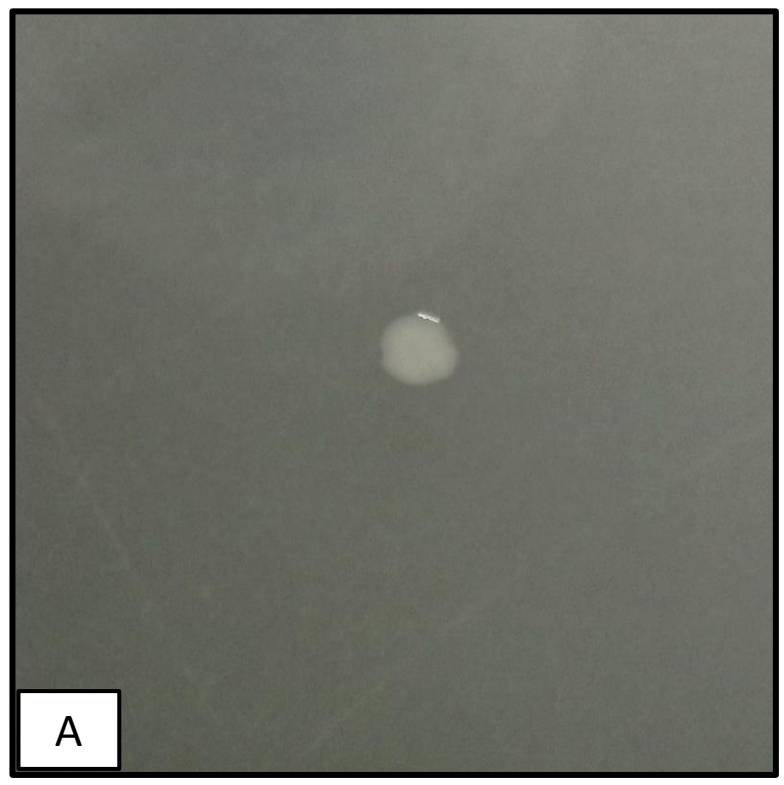
The detection of nitrogen-fixing bacteria was done using a Jensen medium. Jensen medium being nitrogen limiting only supports the growth of nitrogen-fixing bacteria that can utilize atmospheric nitrogen gas. The bacterial isolates were inoculated on Jensen media and after 24-48 hrs, both B1 and B2 strains were observed to be growing indicating both had nitrogen-fixing ability (Figure 4.6 A and B).

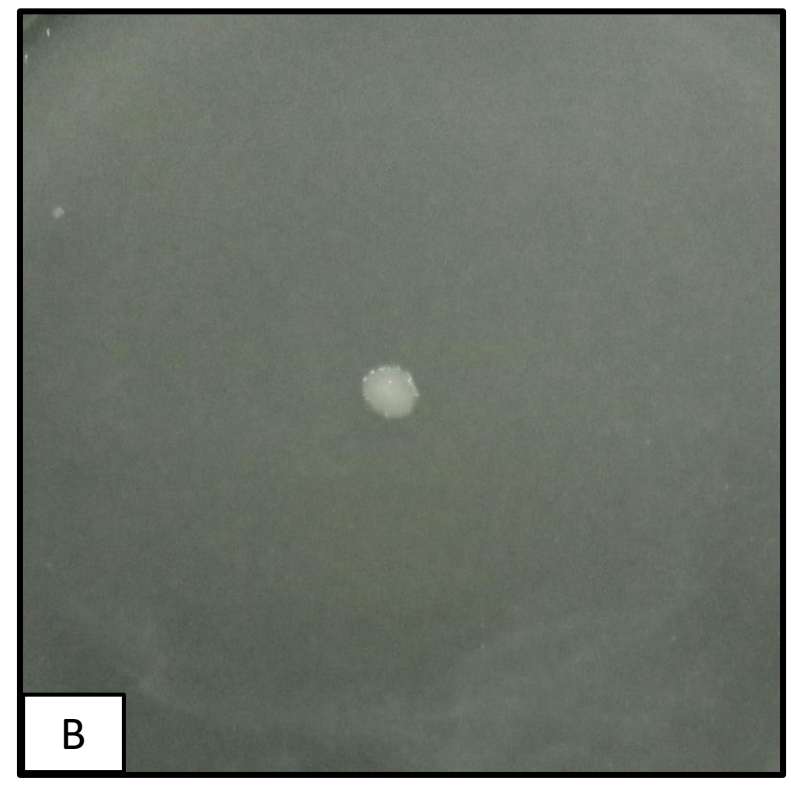
4.8 Plant Growth Promoting Traits of Siderophore Producing Bacteria at High Concentrations of Lead and Zinc.

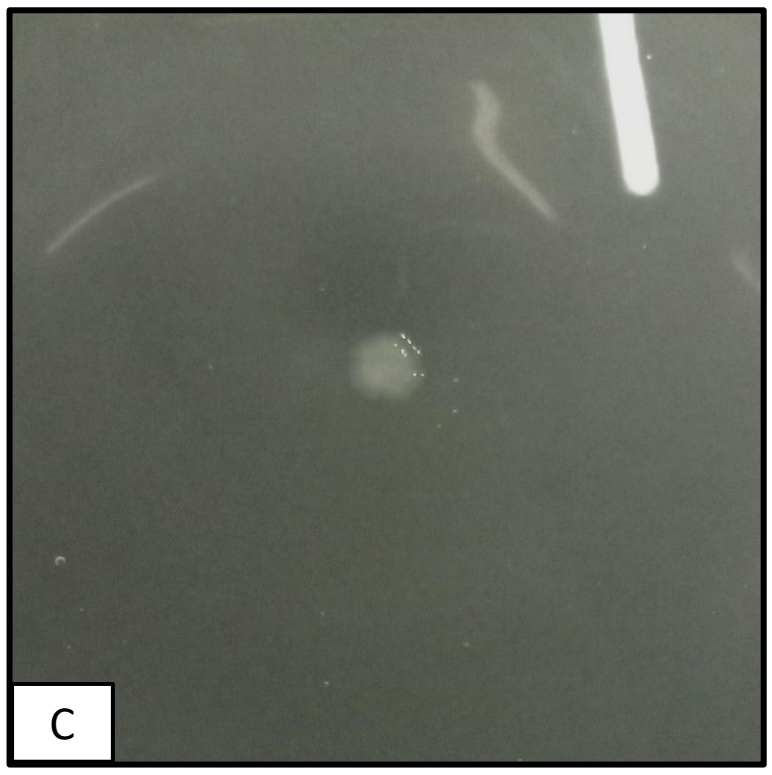
The bacterial isolated B1 and B2 were assessed for their siderophore-producing, phosphate-solubilizing and nitrogen-fixing ability under metal stress conditions. To evaluate siderophore production, phosphate solubilization and nitrogen fixation CAS-agar medium, Pikovskaya medium and Jensen medium were used respectively. Lead (Pb) and Zinc (Zn) were added individually at concentrations of 300 mg Pb/L and 200 mg Zn/L to each medium. Both bacterial strains B1 and B2 were inoculated onto each of the six different treatments (CAS-Agar + Pb, CAS-Agar + Zn, Pikovskaya + Pb, Pikovskaya + Zn, Jensen + Pb and Jensen + Zn).

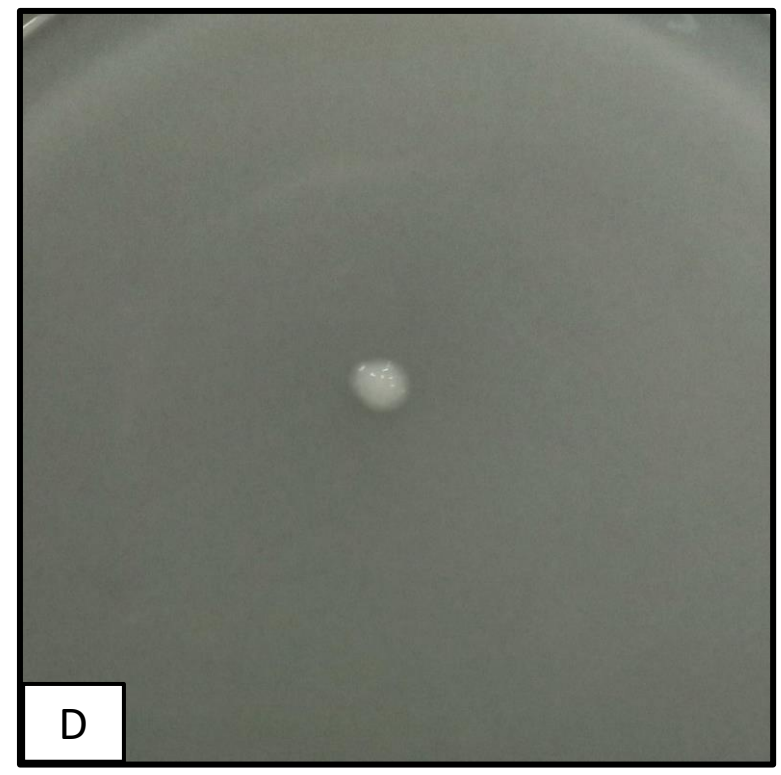
In the case of CAS-agar medium after the incubation of 7 days, it was observed that B2 exhibited a larger halo diameter than B1 in CAS-Agar plates containing Pb (Figure 4.2 C and D). Similarly, B1 showed a smaller halo in Zn compared to B2 in CAS-Agar plates containing Zn (Figure 4.2 E and F). The ratio of halo diameter/colony diameter was seen highest in the case of B2 at 1.84 while B1 showed 1.33 in the presence of Pb and B2 showed the highest ratio at 1.54 while the lowest 1.24 was observed in the case of B1 in the presence of Zn (Table 4.1) (Figure 4.7 A).

In the case of the Pikovskaya medium after incubation of 7 days, it was observed that neither B1 nor B2 showed any clear halo around the colony in the Pikovskaya medium containing Pb (Figure 4.5 C and D) with phosphate solubilizing index as 1 for both. While B1 did not grow









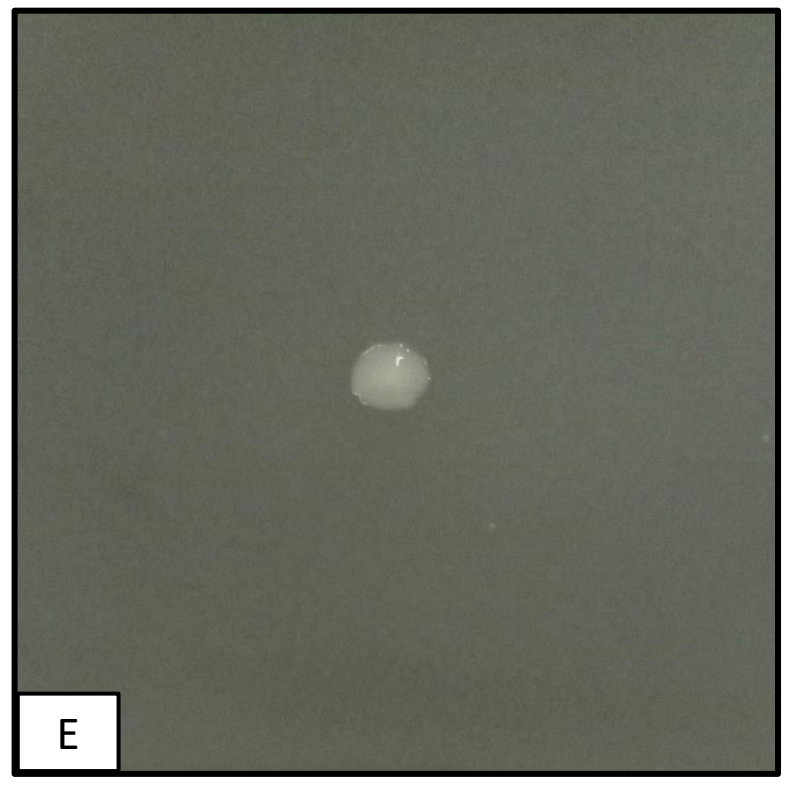
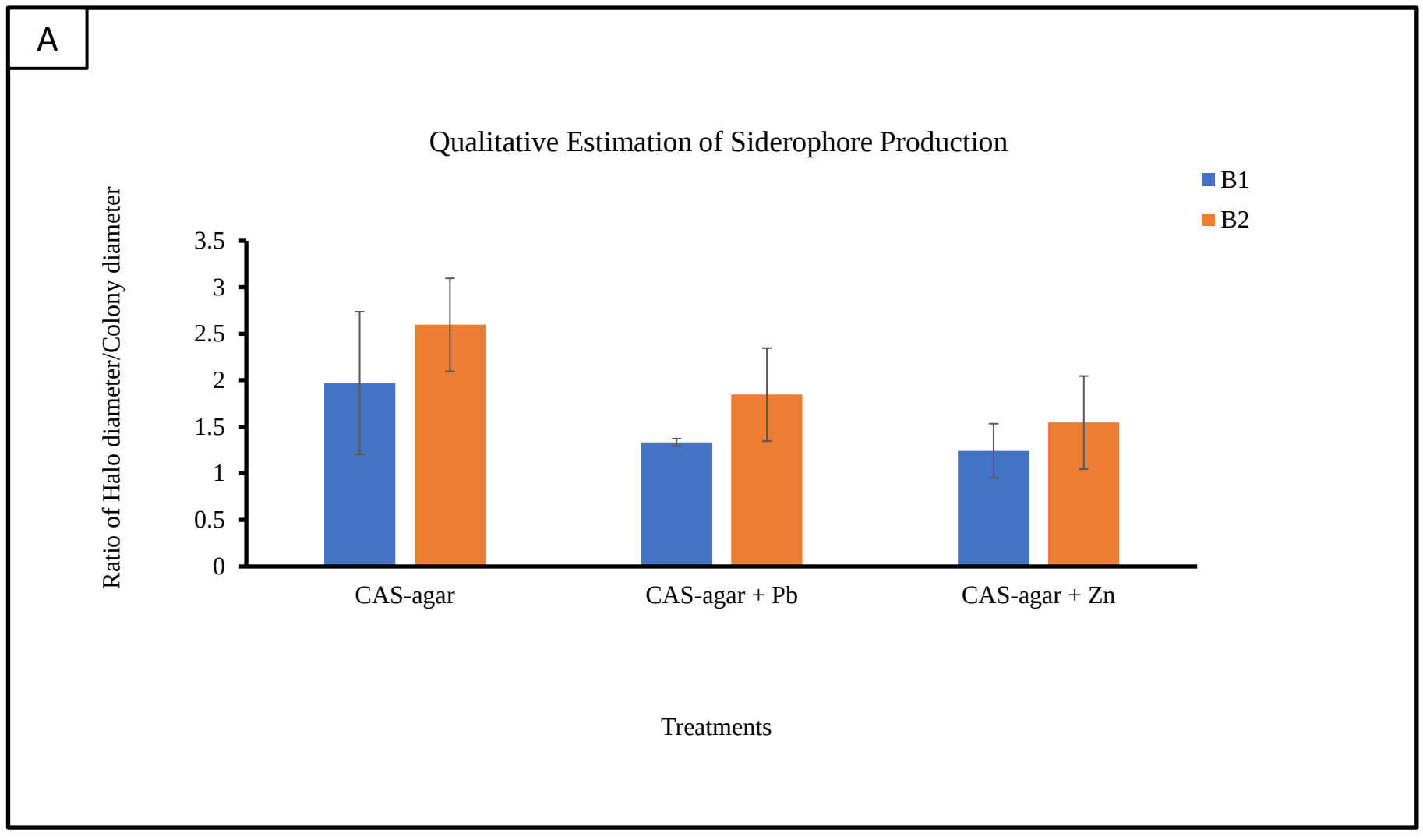


Figure 4.6 Nitrogen-fixing on Jensen plates is indicated by the growth of the bacterial colony. (A) B1 on the Jensen plate, (B) B2 on the Jensen plate, (C) B1 on the Jensen plate containing Pb, (D) B2 on the Jensen plate containing Pb, and (E) B2 on the Jensen plate containing Zn.



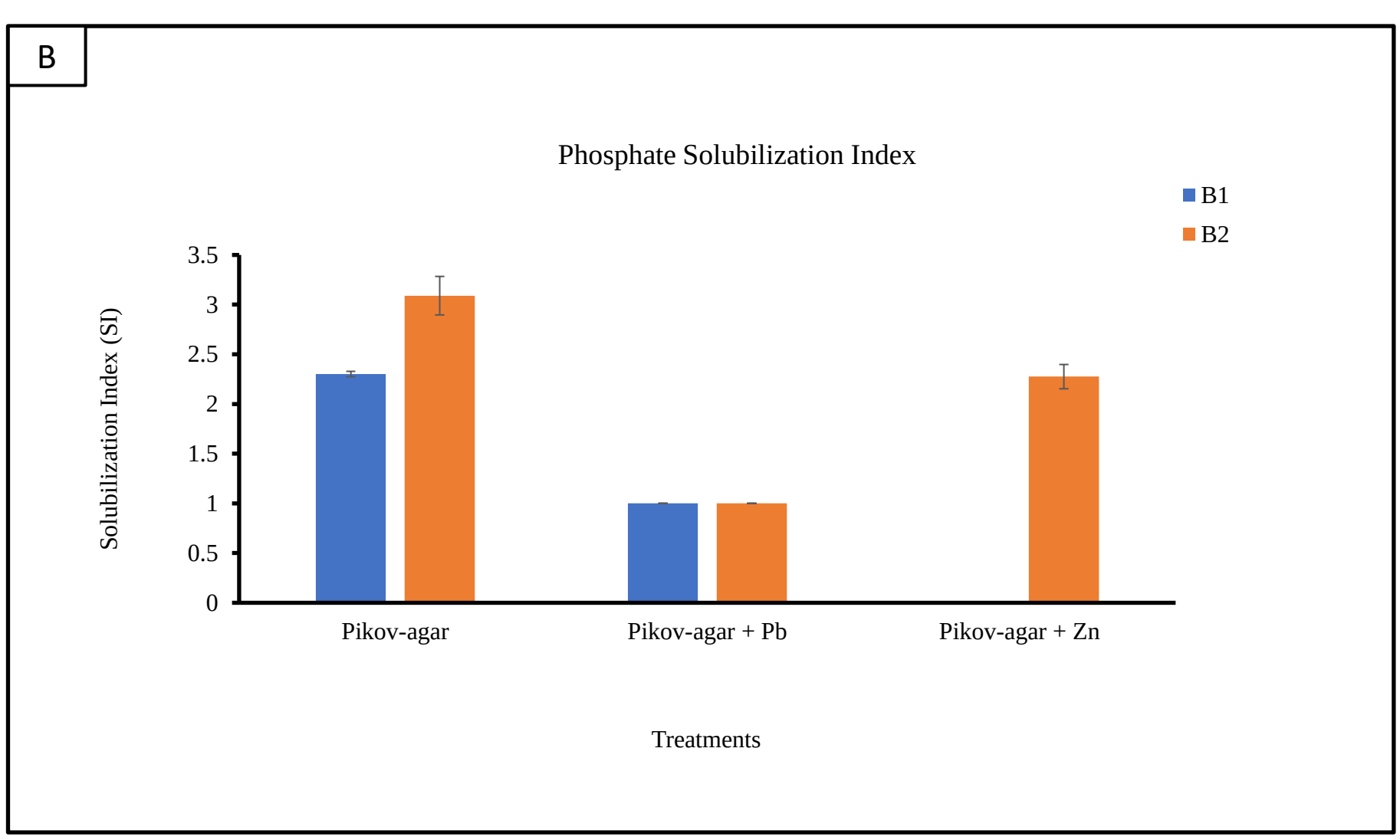


Figure 4.7 Determination of siderophore production and Phosphate solubilization of bacterial isolates B1 and B2. (A) Qualitative estimation of siderophore production and (B) Phosphate Solubilization Index.

when inoculated on Pikovskaya medium containing Zn, B2 exhibited a hazy or slightly clear zone (Figure 4.5 E). The phosphate's highest solubilizing index was found to be 2.27 in case B2 under the presence of Zn (Table 4.1) (Figure 4.7 B).

In the case of the Jensen medium the bacterial growth was checked after 24-48 hrs of incubation, and it was observed that B1 and B2 were found growing in the Jensen medium containing Pb (Figure 4.6 C and D). B1 did not show growth in the Jensen medium containing Zn, whereas B2 exhibited growth (Figure 4.6 E).

Based on these observations we can draw conclusions that B2 outperformed B1 in siderophore production under Pb stress. B1 exhibited reduced siderophore production in Pb and Zn compared to B2. Neither strain showed significant phosphate solubilization in the Pikovskaya medium containing Pb. B1 failed to grow in the Pikovskaya medium containing Zn, while B2 showed limited growth. Both strains grew in the Jensen medium containing Pb. B1 did not grow in the Jensen medium containing Zn, whereas B2 thrived (Table 4.1).

4.9 Tolerance of siderophore-producing bacteria towards different antibiotics

The siderophore-producing bacterial strains B1 and B2 were exposed to three different antibiotics ampicillin, streptomycin and kanamycin by inoculating them onto nutrient agar media containing 100µg/mL each of the antibiotic. After 24-28 hrs of incubation, it was observed that B1 and B2 exhibited growth on media containing ampicillin and streptomycin. Neither strain showed any growth on media containing kanamycin. This suggests that B1 and B2 were resistant to ampicillin and streptomycin while both were susceptible to kanamycin (Figure 4.8 A, B, C and D).

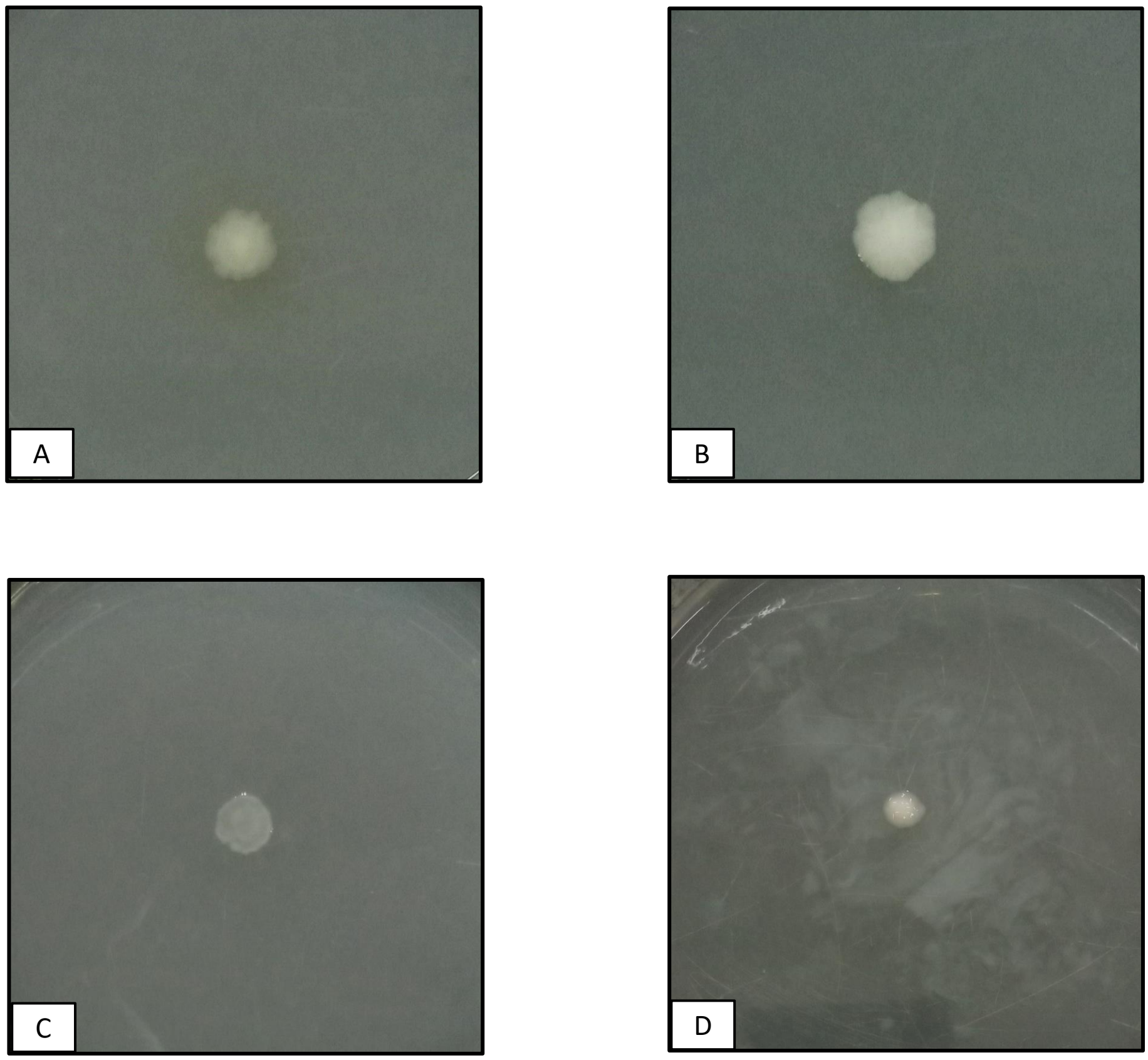


Figure 4.8 Tolerance of siderophore-producing bacteria to different antibiotics represented by bacterial growth on antibiotic-containing medium. (A) B1 on the Nutrient agar plate containing ampicillin, (B) B2 on the Nutrient agar plate containing ampicillin, (C) B1 on the Nutrient agar plate containing streptomycin and (D) B2 on the Nutrient agar plate containing streptomycin.

4.10 Determination of plant growth promotion by the selected bacteria in the presence of lead and zinc

The effects of B1 and B2 bacterial strains on plant growth promotion in contaminated soil with lead and zinc were studied (Figure 4.9). A total of nine different treatments were carried out as listed above in the procedure. Physiological parameters like shoot length and root length were measured in cm before bacterial treatment i.e. after 7 days of growing seedlings and after 7 and 14 days of bacterial treatment. The trend in the increase of shoot length and root length was quite similar in both the 7^{th} and 14^{th} day of bacterial treatment hence 14th-day observations were considered for comparison between the treatments.

Treatment B1 showed an increase in the shoot length and root length by 8% and 11% than the control respectively (Figure 4.10), Treatment B2 showed a 15% increase in the shoot length compared to the control while there was no significant difference observed in the root length. This indicates that these bacteria promote plant growth. Treatment Pb showed a decrease in the shoot length and root length when compared to the control by 7% and 3% respectively. In the case of treatment, B1+Pb showed a 3% decrease and B2+Pb showed a 6% increase in the shoot length when compared to the control but showed 13% and 16% increases in the shoot length when compared to treatment Pb, which indicates that inoculating the Pb contaminated soil with B1 and B2 showed increase in the plant height than the plant in Pb contaminated soil which were uninoculated with any bacteria. Treatment Zn showed a decrease in the shoot length and root length when compared to the control by 16% and 17% respectively. Treatment B1+Zn and B2+Zn also didn't show much improvement in shoot length when compared to the control treatment but a 24% and 11% increase in the shoot length were observed when compared to the treatment Zn. Enhanced root length of about 6% of treatment B1+Zn was observed when compared to Zn treatment. The plants of the treatment Pb and Zn showed reduced shoot and

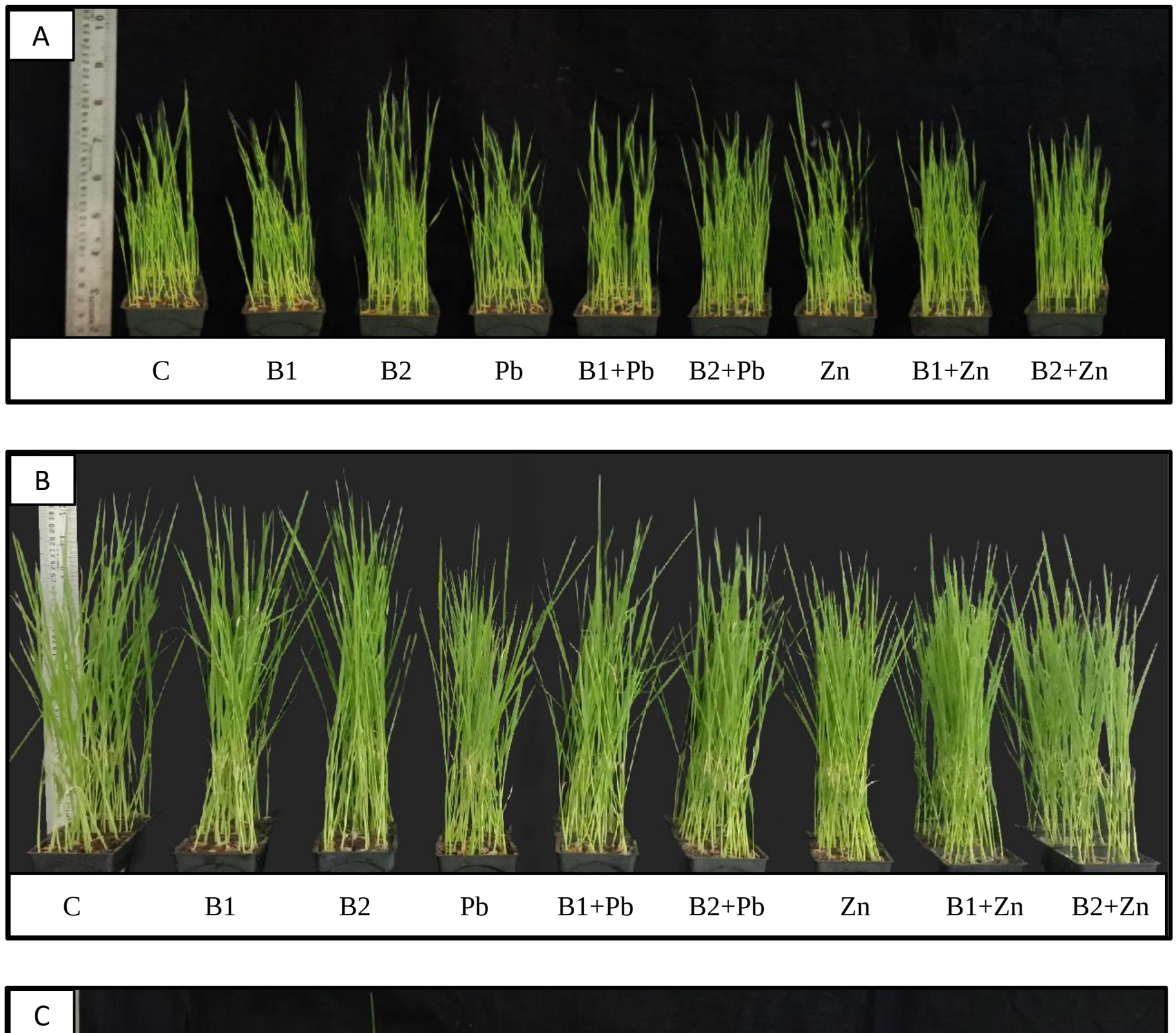


Figure 4.9 Growth of IR64 Rice seedlings under different treatments. (A) Before bacterial treatment, (B) After 7 days of bacterial treatment and (C) After 14 days of bacterial treatment.

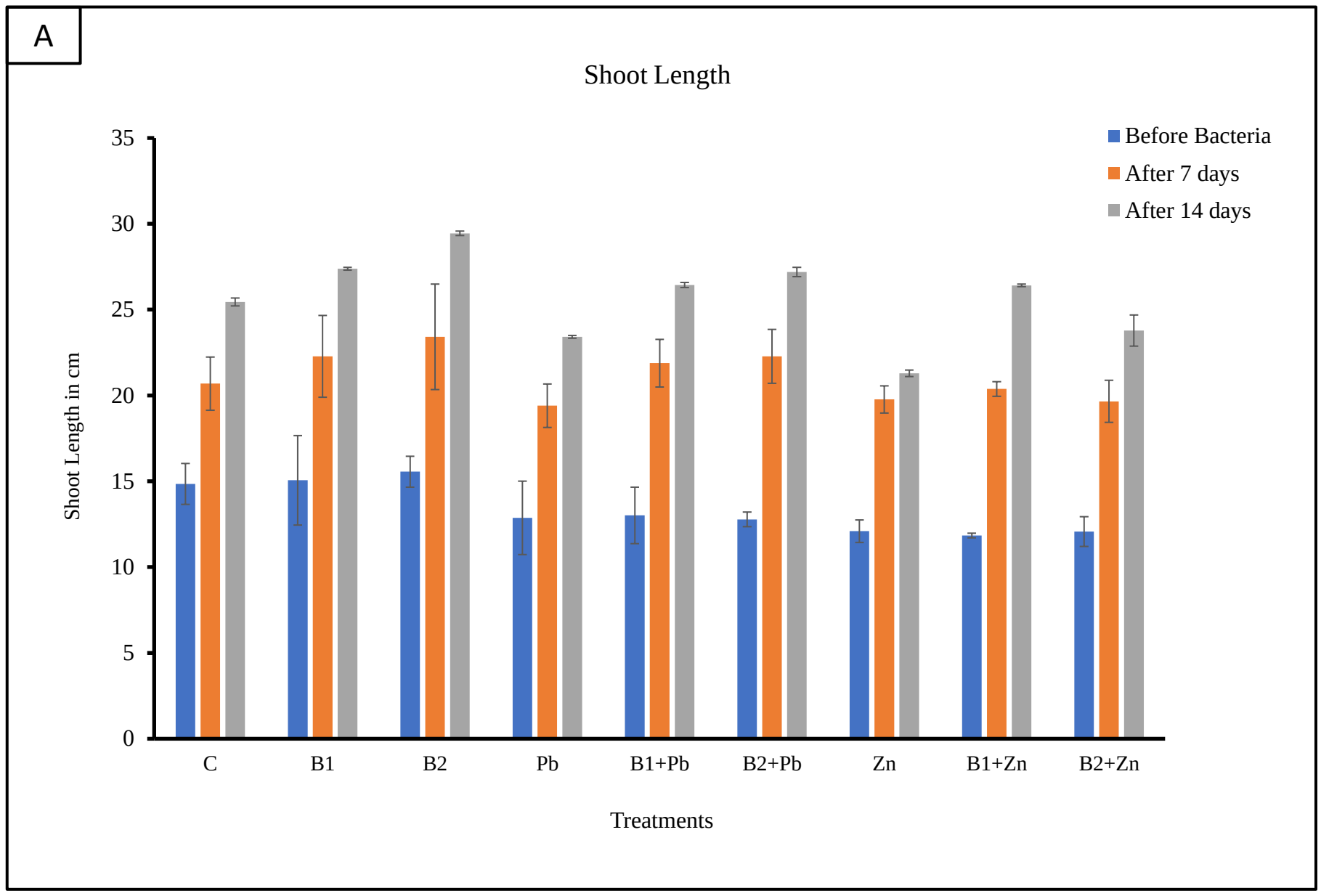


Figure 4.10 The effects of B1 and B2 bacterial strains on plant growth promotion in contaminated and uncontaminated soil with Pb and Zn. (A) Shoot Length. and (B) Root Length.

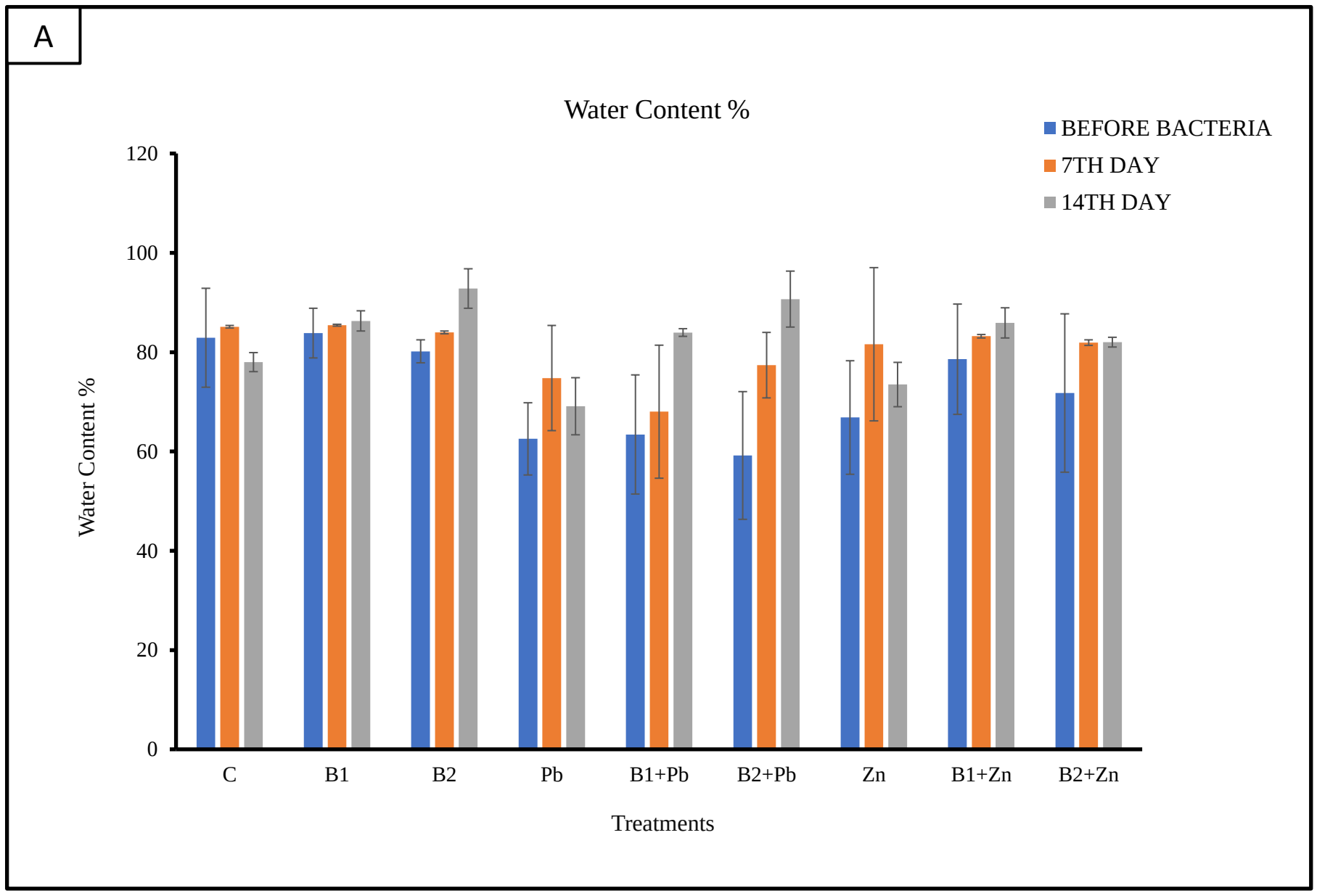


Figure 4.11 Effects of B1 and B2 bacterial strains on Water content % of plants grown in contaminated and uncontaminated soil with Pb and Zn.

Table 4.3 Effect of B1 and B2 bacterial strain on shoot length, root length and water content %

TREATMENT	SHOOT LENGTH (cm)		ROOT LENGTH (cm)		WATER CONTENT %	
	Before bacteria	After 14 days	Before bacteria	After 14 days	Before bacteria	After 14 days
C	14.84±1.19	25.44±0.23	7.28±0.18	8.91±0.49	82.91±9.9	78±1.91
B1	15.05±2.60	27.37±0.08	8.2±0.98	9.91±0.38	83.84±4.9	86.29±2.02
B2	15.55±0.90	29.44±0.12	7.82±0.28	9.15±0.21	80.15±2.31	92.84±3.97
Pb	12.86±2.14	23.41±0.08	6.52±1.07	8.65±0.25	62.55±7.26	69.1±5.74
B1+Pb	13.01±1.64	26.43±0.143	6.54±1.33	8.82±0.35	63.43±12.00	83.95±0.75
B2+Pb	12.77±0.42	27.18±0.26	6.37±0.51	8.78±0.52	59.2±12.85	90.69±5.63
Zn	12.08±0.65	21.28±0.19	5.73±1.11	7.41±0.62	66.84±11.42	73.48±4.46
B1+Zn	11.83±0.13	26.41±0.076	5.96±0.26	7.86±0.63	78.59±11.10	85.9±3.05
B2+Zn	12.06±0.86	23.77±0.90	5.92±0.75	7.47±0.27	71.77±15.95	82.02±0.96

Data is presented as means of 3 replicates ± SD (standard deviation).

root length due to the high concentration of lead (Pb) and zinc (Zn). Table 4.3. shows the average shoot and root length in cm ± standard deviation.

Another parameter water content % was determined (Figure 4.11). Percentage Water content of treatments B1 and B2 showed a 10% and 19 % increase than the control. Treatment Pb, Zn and B1+Pb showed a decrease in the water content % by 11%, 5% and 7% respectively, while treatment B2+Pb markedly improved water content % to 16% than the control. Treatment B1+Zn and B2+Zn also showed significant increases of about 10% and 5% respectively than the control. But when the water content % was compared with the treatment Pb an increase of 21% and 31% was noticed in the case of B1+Pb and B2+Pb respectively. When the water content % was compared with the treatment Zn an increase of 16% and 11% was noticed in the case of B1+Zn and B2+Zn respectively (Table 4.3).

CHAPTER 5: CONCLUSION

Heavy metals (HMs) are among the most widespread soil contaminants worldwide and their presence is reported in 60% of polluted land. The presence of heavy metals at concentrations higher than natural ones poses a risk because of their toxicity. Siderophores are low molecular weight compounds (between 500 and 1500 Dalton), of high affinity and selectiveness to bind and complex Fe (III). In both bacteria and plants, they can enhance the uptake of iron, an essential micronutrient. The importance of siderophores is obvious, and they play a significant role in environmental applications, The current study was framed to explore siderophore-producing bacteria which are metal tolerant having the ability to chelate metal ions for improving plant growth under high metal toxic environments. Two potential bacterial strains were isolated from the rhizosphere of the *Jaya* rice plant named B1 and B2 growing in fields near mining areas affected by mining effluents. In addition to their high tolerance to Pb (300mg/kg) and Zn (200mg/kg) and siderophore production, these strains also exhibited various plant growth-promoting activities such as phosphate solubilizers and nitrogen fixation. To evaluate the extent of PGPR attributes under Pb and Zn toxicity rendered by the LTB and the ZTB, IR64 rice plants were inoculated with the strains. There was a noticeable increase in the shoot length, root length and percentage of water content when plants were inoculated with individual bacteria B1 and B2 as compared to the control. The decreased growth of Pb and Zn-stressed plantlets was possibly attributable to the activation of plant defence mechanism and also to the reduced synthesis of plant growth-promoting substances. However, the application of LTB and ZTB strains significantly improved the growth i.e. shoot length, root length and percentage of water content of rice plantlet under Pb and Zn stress conditions. However, the increase in shoot length, root length and water content of the plants grown in Pb and Zn-contaminated soil was less as compared to the control treatment. The results indicated that the LTB and ZTB can be used as microbial inoculants for improving agriculture in Pb and Zn

-contaminated soil and bioremediation of heavy metals in polluted industrial sites. Further to confirm the efficacy of these bacterial strains, dedicated further studies are required on different crops under Pb and Zn stress conditions for the determination of Pb and Zn bioremediation potential of these isolates. Further research focusing on finding effective ways to use siderophores in bioremediation and as biofertilizers needs attention which would enhance their application in the environment.

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