NITROGEN FIXATION EVALUATION OF *AZOTOBACTER CHROOCOCCUM* ISOLATED FROM SOIL OF FOREST

Dissertation
Submitted to the **Department of Microbiology,**
Central Campus of Technology, Tribhuvan University, Dharan, Nepal,
in Partial Fulfillment of the Requirements for the Award of Degree of
Master of Science in Microbiology
(Agriculture)

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This is to certify that **Miss Priskila Tolangi** has completed this dissertation work entitled **"NITROGEN FIXATION EVALUATION OF *Azotobacter chroococcum* ISOLATED FROM SOIL OF FOREST"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (**Agriculture**) under my supervision. To my knowledge, this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Hemanta Khanal** this dissertation work of **Miss Priskila Tolangi** entitled **"NITROGEN FIXATION EVALUATION OF *Azotobacter chroococcum* ISOLATED FROM SOIL OF FOREST"** has been approved for the examination and is submitted for the Tribhuvan University in partial fulfillment of the requirements for M.Sc. degree in Microbiology(Agriculture).

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ABSTRACT

With the continuous use of chemical fertilizers in Agriculture system may help to produce sufficient amount of food for growing population but these chemicals have an adverse effect both on the environment and living organisms. Biofertilizers are a promising alternative to hazardous chemical fertilizers and gaining importance for attaining sustainable agriculture. They play key role in maintaining soil health and plants. Microorganisms can interact with the crop plants and enhance their immunity, growth, and development. Nitrogen, Phosphorous, Potassium are the essential nutrients required for the growth of crops, but these nutrients are naturally present in insolubilized forms. Certain microorganisms make them soluble and can be easily assimilated by plants for their growth and development. In this study, *Azotobacter chroococcum* were isolated from forest surroundings of Panbari Dharan. 25 soil samples were collected in sterile bag and cultured in Ashby's media by serial dilution and incubated at 30˚C for 5 days. Morphological, cultural, biochemical characterization was done and bacteria were identified. Isolated *A. chroococcum* was cultured in Ashby's broth and inoculated on tomato seedlings by root dipping technique. It was found that tomato plants inoculated with *A. chroococcum* has significant growth in height, no. of leaves and root length than that of untreated tomato plants. The research result showed that this strain had positive effect on the increase of nitrogen content of soil. On the base of this study, it is concluded that *Azotobacter chroococcum* has positive effect in growth of plants. It could be good biofertilizer as an alternative to nitrogen fertilizer.

Keywords: soil, free-living nitrogen fixers, nitrogen, PGPR effects, tomato plants

LIST OF ABBREVIATIONS

ANOVA	:	Analysis of Variance
PGPR	:	Plant growth promoting rhizobacteria
S-NI	:	Sterile-non inoculum
NS-NI	:	Non sterile- non inoculum
S-I	:	Sterile-inoculum
NS-I	:	Non sterile-inoculum
DAT	:	Days After Transplantation
NA	:	Nutrient Agar
IAA	:	Indole acetic acid
NPK	:	Nitrogen Phosphorus Potassium
ATP	:	Adenosine triphosphate

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CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Nitrogen is one of the most vital factors needed for plant development and proscribing element in plant growth. It represents approximately 2% of the overall plant dry matter that enters the food chain. Nevertheless, plants cannot directly take nitrogen gas, which covers up about 78% of the atmosphere. Plants consume the available nitrogen in the soil via their roots in the form of ammonium and nitrates. (Dobermann 2007, Paterson, Bowers et al. 2009). Plants require nitrogen (N) in larger amounts than any other mineral nutrient, and are generally the most growth-limiting factor (Weetman, Prescott et al. 1997, Nohrstedt 2001, Saarsalmi and Mälkönen 2001, Paterson, Bowers et al. 2009).

At present, about 60% of artificial nitrogen fertilizers are used for cereals, with irrigated rice manufacturing accounting for approx. 10% of the use. Since 50% of the fertilizers implemented are mainly used by plants, the inefficient use of nitrogen results in nitrate contamination of soils and ground water, which reasons health risks and compromising agricultural sustainability. Moreover, for the manufacturing of N fertilizer requires six times extra energy than that had to produce both P or K fertilizers.(Sun, Ricardo-da-Silva et al. 1998).

Chemicals fertilizers had been used considerably to rise crop production in recent days. Extensive use of chemical fertilizers in agriculture may also make a country self-enough in food production, however chemical compounds have negative effects each at the surroundings and dwelling organisms. In addition, the chemical fertilizers are costly to acquire by the farmers, have an effect on soil, reduce its water-retaining ability and fertility, cause imbalance within the soil nutrients, and bring about unacceptable ranges of water pollution (Sprent and Sprent 1990).

Plants offer a multitude of niches and habitats for the better growth and proliferation of a mess of microorganisms, which includes bacteria, fungi, protists, nematodes , and viruses. These soil microorganisms have complicated co-associations with plants. They play essential roles in the production and healthiness of the plant in natural habitats (Lundberg, Lebeis et al. 2012, Cregger, Veach et al. 2018)

The members of plant microbes donates beneficial, neutral and pathogenic microorganisms. Plants microbes related to their hosts were shown to enlarge plant growth and development, uptake of nutrient, and disease resistance (Richardson and Simpson 2011, Gouda, Kerry et al. 2018). The advantages of the microorganisms to their host plants may be direct, which include transformation and translocation of main nutrients in the soil to make them aaccessible to plants life (for example, nitrogen fixation, phosphorus solubilization), mitigation of stressful environment (such as drought) and safety from plant pathogens via competition, antibiosis and the manufacturing of hydrolytic enzymes (Trivedi, Trivedi et al. 2016, Gouda, Kerry et al. 2018). The rhizosphere comprises the major source of microorganisms, in which root exudates consist of enzyme, water, and carbon compounds that move into plants through the root and set up sub-communities inside plants (Hardoim, Van Overbeek et al. 2015). Consequently, the root has appeared as a hot spot of plant-microbial interactions (Sessitsch and Mitter 2015).

Microorganisms which are mainly called biofertilizers when implied to seed, plant surfaces, or soil, colonize the rhizosphere or the internal part of the plant and help for the growth and development by the aid of supplimentation or availability of basic nutrients to the host plant (Vessey 2003). Biofertilizers come among the specific biological processes and are taken into consideration as an interesting active microbial task on the earth's surface because it affords a way of improvement of nitrogen and performs vital role in nitrogen homeostasis withinside the biosphere (Wani, Kumar et al. 2016). In agriculture, vegetation required a large amount of the nitrogen to absorb as a nutrition in soil as they grow and this causes the soil to lose a lot of nitrogen every year (Akhtar and Siddiqui 2008).

In fact, BNF is said to be a natural way of converting atmospheric nitrogen into the simplest soluble non-toxic form (NH4+ primarily) which can be taken up by plant cells for production of different biomolecules. BNF is one of the most essential sources of nitrogen for crops and an important step dispensing this nutrient in the ecosystem (Saikia and Jain 2007, Sur, Bothra et al. 2010). Nitrogen input through BNF can help to maintain healthy soil as well as attain large crop yields(Peoples and Craswell 1992).

Nitrogen-fixing diazotrophs could be classified into three categories, they may be free-living nitrogen-fixing bacteria, symbiotic nitrogen-fixing bacteria, and associative nitrogen-fixing bacteria (Zhou 2017). These nitrogen-fixing bacteria produce nitrogenase enzymes and provide the anaerobic environment for nitrogen fixation. After the fixation, nitrogen enters the biosphere and is utilized for biomolecule synthesis (Zhu and Chen 2002). Free-living N-fixers may be located in soil, water, rhizosphere, and leaf surfaces. Photoautotrophs are those organisms that rely upon light for energy. Heterotrophic N fixers are other groups of diazotrophs. However, they are typically limited in their fixation capacity because of a shortage of organic substrates to generate energy (Russelle 2008). Among the heterotrophic free-dwelling N2-fixing organism, belonging to the genera *Azotobacter* is one of the most investigated genera which are able to fix atmospheric nitrogen into ammonia and nitrates withinside the soil which can be utilized by plants life for their growth and development.

Besides its capability for the fixation of atmospheric nitrogen, *Azotobacter* also synthesizes biologically active growth-supporting compounds such as indol acetic acid, gibberellins, and B-vitamins in culture media that benefits plants in their growth (Thakur and Sharma 2005). *Azotobacter* has played a remarkable role, being broadly dispersed in different environments, such as soil, water and, sediments (Palleroni 1984). In fact, field trials have demonstrated that under certain environmental conditions, inoculation with *Azotobacter* has beneficial effects on plant yields (Rovira 1969, Brown 1974, Mrkovacki and Milic 2001). *Azotobacters* are known to be highly important for their ability to fix molecular nitrogen, contributing to the productivity of

plants. They were proved experimentally to fix 10 mg of atmospheric nitrogen per gram of carbohydrate consumed (Becking 1992).

*Azotobacter*s are free-living, aerobic, heterotrophic bacteria. The bacteria are gram-negative, and lie in the class γ-proteobacteria. They are oval or round in form and have thick-walled cysts (dormant cells against deleterious conditions) a number of which might be motile through peritrichous flagella while others are immotile (Martyniuk and Martyniuk 2003). They are usually polymorphic having a length ranging from 2 to 10 mm long and 1 to 2 mm wide. The genus *Azotobacter* was identified in 1901 by a Dutch microbiologist, botanist and founder of environmental microbiology-Beijerinck and his coworkers found out first aerobic free-living nitrogen fixer. These bacteria are recognized to spread atmospheric nitrogen for protein synthesis in their cells that are mineralized in the soil, supplying the crop plants a significant part of nitrogen available from the soil source (Zulaika, Shovitri et al. 2014).

The first species of *Azotobacter* was described as *A. chroococcum*. Other species are defined as *A. vinelandii, A. beijerinckii, A. nigricans* , *A. armeniacus* and, more recently *A. salinestris.* The study of detailed taxonomic and ecology of the *Azotobacteraceae* analyzing many morphological, physiological, and biochemical details of the aerobic N2-fixers was conducted by(Thompson 1987). The distribution of ecology of *Azotobacter* spp. is a complex concern and is associated with numerous factors which decide the presence or absence of this bacterium in specific soil. It has been proven that the soil properties and climatic conditions have an effect on the distribution of this microorganism (Magalhães Cruz, Maltempi de Souza et al. 2001, Tejera, Lluch et al. 2005). It consists of natural organic matter content, moisture, C/N ratio, and pH (Tejera, Lluch et al. 2005).

As mentioned earlier that the production of plant growth regulates such as phytohormones and vitamins is a basic property among *Azotobacters* (Arshad and Frankenberger 1991) in which they help in plant root proliferation, respiratory rate and metabolism, enhancing mineral, and water uptake in plants inoculation (Okon and Itzigsohn 1995). It has been reported that *Azotobacter*

strains also have antifungal properties, and thus suppressing of pathogenic fungi enhancing the better growth of plants. So, this bacteria have been often discussed as a mechanism that benefits plant growth (Rauschkolb, Brown et al. 1974). However, the conditions of environment affects the performance of BNF, suggesting that indigenous nitrogen-fixing bacteria may achieve better adapt nearby niches than inoculated biofertilizers for regional crops (Toledo, Gonzalez-Lopez et al. 1988, Kannan and Ponmurugan 2010).

Azotobacter particularly *A. Chroococcum* is spread widely in agricultural soils of temperate areas with neutral or alkaline soils, and in addition, they may be easily discovered in relation to cereal and legume rhizospheres (Orozco-Ramírez and Astier 2017, Mandal, Dutta et al. 2019).It has been reported in many studies that *A. chroococcum* as soil inoculant is not always most effective in Nitrogen fixation but additionally has different characteristics such as the production of growth hormones (Cave 2000), production of fungicidal substances, siderophsore production, and the property to solubilize phosphate in the soil. (Kumar and Narula 1999, Singh, Rao et al. 2007). Research done on *Azotobacter chroococcum* in crop production has found its main reason in the improvement of plant nutrition and the amelioration of soil fertility (Murray-Tortarolo, Jaramillo et al. 2018).

Azotobacter spp. has additionally been reported to decompose a wide range of other chlorinated phenols like 2-Chlorophenol, 4- Chlorophenol, 2,6-Dichlorophenol, and 2,4-6-Trichlorophenol (Wang, Gao et al. 2009). *A.chroococcum* notably metabolized 2,4-dichlorophenoxyacetic acid (2,4-D) as the main source of carbon (Arunachalam, Raj et al. 2003, Singh, Kumar et al. 2016). *A.chroococcum* is also capable of remodeling a famous herbicide, pendimethalin into non-poisonous products, concluding the fact that the bacterium is very important not only for effective crop production but also for the safety of the environment (Wasi, Tabrez et al. 2013).

Maize plants inoculated with *Azotobacter spp*. have been found to enhance growth in control and saline stress conditions. Research on wheat plants with inoculation of *A.chroococcum* showed improved phosphorous nutrition, rise in grain mass and root biomass, increased level of osmotic adjustment and

activation of ROS response genes (Kumar, Behl et al. 2001). Among the various species of *Azotobacter* like *A. vinelandii, A. insignis, A. beijerinckii, and A. macrocytogenes, A. chroococcum* is the most prevalent species determined in the soil (Eklund, Degerald et al. 2017) reported that the presence of *A. chroococcum* in the rhizosphere of cucumber and tomato turned into increased growth and germination of seedlings. In current years, microbial inoculants which contain N2-fixing and other useful microorganisms have experimented on potted plants, nurseries, and field crops. (El-Sirafy, Woodard et al. 2006, Aseri, Jain et al. 2008). Hence, the main objective of this experiment is to characterize nitrogen-fixing bacteria *Azotobacter chroococcum* and to evaluate nitrogen fixation, and PGPR effects of this bacterium on tomato plants.

1.2 Objectives

1.2.1 General Objectives

- Nitrogen fixation evaluation of *Azotobacter chroococcum* isolated from soil of the forest.

1.2.2 Specific Objectives

- To isolate and characterize the free living bacteria (*Azotobacter chroococcum)* from the soils of the forest surrounding Dharan.
- To evaluate the difference in nitrogen contents and to study the PGPR effects of *Azotobacter chroococcum* inoculated in tomato plants.

CHAPTER II

LITERATURE REVIEW

2.1 Impact of chemical fertilizers

According to the United Nations Food and Agriculture Organization (FAO) projections, the total population around the world will attain at least 9.8 billion by 2050 (Onopriienko and Kharytonov 2019). To ensure global food security, we have to increase our current agricultural production (Conforti, Ahmed et al. 2018). Different crop nourishment strategies are being explored by farmers. To date, chemical fertilizers assist in feeding the global by contributing three main important plant nutrients, nitrogen, phosphorus, and potassium (NPK). Approximately 52.3 billion tons of P-based fertilizers are used yearly to manage available P levels in soil-plant systems (Bruinsma 2017). Whereas, most approximately 0.2%, i.et., <10μM of this large amount, is consumed by plants (Alori, Glick et al. 2017, Peng, Chen et al. 2019), and the remaining is prompted with the aid of metal cation in soil which include Fe, Al, Mg, Ca, etc (Leghari, Laghari et al. 2016).

Increasing the use of chemical fertilizers in the Agriculture system may help to produce a sufficient amount of food for the increasing population however these chemical compounds have a negative effect both on the surrounding environment and living organisms. They have an adverse effect on the soil, lessen its water-holding capacity and fertility, which result from imbalance withinside the soil nutrients, and brings about unacceptable degrees of water pollution (Sprent and Sprent 1990). Due to the excess use of agrochemicals, the global climate is experiencing a drastic change in soil results the depletion of essential nutrients present in thesoil. (Sujanya and Chandra 2011) reported that the extensive use of chemical fertilizers in agriculture help to build the country self-dependent in offering a massive quantity of food delivered but simultaneously destroy the environment and causeing harmful impacts on living beings. The increasing uses of chemical fertilizers have a considerable risk to ecosystem by polluting air, water, and soil (Saxena, Rana et al. 2013).

Since those hazardous chemical components cannot be consumed by the plants, they begin collecting in groundwater and many of these kinds of chemicals are also accountable for bringing eutrophication of water sources (Konotop, Mezsaros et al. 2012).

Considering all of the negative consequences of continuous consumption of chemical fertilizers concerning our health, organic farming has been evolved as a potent alternative method in terms of the increasing demand of healthy food supply, long-term sustainability, and concerning environmental pollution (Awasthi and Reddy 2013). Recently many researchers from everywhere in the world have been concerntrating on long-term agriculture by using beneficial microorganisms so that it will add the biofertilizer to food and fiber production (Ahmed, Uddin et al. 2007, Granada, Passaglia et al. 2018, Yadav and Sarkar 2019). Many species of microorganisms, a number of which have exciting biotechnological capability were isolated from the wide range of research programs on plant-useful microorganisms (Stella and Sivasakthivelan 2009, Kaur and Purewal 2019, Nafis, Raklami et al. 2019).

Microbes play a very significant role in regulating the dynamics of various processes such as the decomposition of organic matter, the accessibility of various nutrients of plants such as iron, magnesium, nitrogen, potassium, and phosphorus, and promoting the growth of the plants (Lalitha 2017) The combinations of nitrogen fixers, potassium and phosphorus solubilizes with molds fungi are commonly utilized as components of biofertilizer (Mohammadi and Sohrabi 2012, Abebe, Pathak et al. 2016). The usage of plant growth supporting rhizobacteria (PGPR) known as biofertilizer in agriculture is continuously growing as they offer an significant tool for the replacement of the use of chemical fertilizers, insecticides, and many other detrimental supplements (Kumar, Verma et al. 2017, Ansari and Mahmood 2019)..

2.2 Nitrogen as an essential element for plant growth

Nitrogen is regarded as essential element required for living creatures (Lemaire and Gastal 1997). Nitrogen (N) is the most abundant element in the

earth after carbon (C), hydrogen (H), and oxygen (O) which is found mainly in the atmosphere (Elser, Bracken et al. 2007). It is one of the main important nutrients for growth and production of crops (Tairo and Ndakidemi 2013). Plants usually need nitrogen (N) in larger amounts than any other mineral nutrient such as potassium (K) and phosphorus (P). In boreal forests, plant-available N is generally the most growth-limiting factor (Weetman, Ajjan et al. 1997, Smolander, Kukkola et al. 2000, Nohrstedt 2001, Saarsalmi and Mälkönen 2001).

In soil, nitrogen can be found in two major forms: inorganic, mineral nitrogen (~2%), and organic (~98%). Inorganic forms include ammonia (NH3), ammonium ($NH4^{+}$), nitrite ($NO2^{-}$), and nitrate ($NO3^{-}$), while organic forms are found in living organic matter (soil biota and fresh animal and plant debris) and non-living organic matter including humified and non-humified compounds. Mineral nitrogen is available to plants in two forms, either as ammonium nitrogen (NH4+-N) or as nitrate-nitrogen (NO3-N). Organic nitrogen is not directly available to plants and must be converted through a slow process (mineralization) to ammonium or nitrate. The main nitrogen sources for the plants were nutrient translocation and litter decomposition while N originating from atmospheric deposition contributed less than 30% of the annual demand (Pihlatie, Christiansen et al. 2013).

Nitrogen is especially linked to diverse crucial life processes in plants including growth, leaf area growth, and biomass-yield productivity. Nitrogen is an important structural part in different kinds of plant molecules such as amino acids, chlorophyll, nucleic acids, ATP, and phytohormones, it is vital component to accomplished the biological processes, which are carbon and nitrogen metabolisms, photosynthesis, and protein production (Frink, Waggoner et al. 1999). Thus, an appropriate amount of nitrogen in soil helps plants in the improvement of root proliferation, volume growth, area, diameter, total and main root length, dry mass and increase subsequent nutrient uptake and improve the nutrient balance and dry mass production (Stitt and Krapp 1999, Diaz, Saliba-Colombani et al. 2006).

Along with these facts, nitrogen also aids greenness of plants, CO2 consumption rate, and crop production and provide resistance to environmental stresses which includes lack of excess amount of water and soil salinity (Bondada, Oosterhuis et al. 1996, Chen, Hou et al. 2010). Moreover, it helps to stimulate rapid early growth, improves fruit quality, enhance the growth of leafy vegetables, and increase the protein content of fodder crops. It also encourages the uptake and utilization of other nutrients including potassium, phosphorous and controls the overall growth of the plant (Bloom 2015, Hemerly 2016). Deficiency in nitrogen uptake in plants can hinder the growth and development followed by the slow development of plant and early leaf senescence which decreased both crop production and quality (Dong, Li et al. 2012).

2.3 Plant-microbes interaction.

The interactions among the plant and microorganisms can be manifold and the advantages of microorganisms on plant health and their growth can be either directly, producing of phytohormone, modulation of ethylene levels in the plant and repression of pathogen growth (Mitter, Petric et al. 2013), or indirectly by inducing modifications in the host plant gene process (Alfano, Ivey et al. 2007, Marrugo-Negrete, Pinedo-Hernández et al. 2017) or changes in microbiome composition (Ardanov, Lyastchenko et al. 2016). The plant itself also influences the composition and activity of its associated microbiota (Sessitsch and Mitter 2015, Lareen, Burton et al. 2016). The soil microbial community play an important role to soil organic matter turnover, nutrient cycling, and plant productivity (Kramer, Dibbern et al. 2016). The main region for plant microbial interactions is in the rhizospheres and root region, where the attraction of microorganisms during drought conditions is mediated by numerous root exudates, by processes that remain largely uncharacterized. Different biotic and abiotic stresses cause a significant decrease in crop growth and productivity and thus are important constraints on global food security. For example, worldwide, annual yield losses caused by plant diseases and pests are estimated at USD $220 billion. (Xiong, Song et al. 2020).

Throughout a previous couple of years, plenty of interest has been directed towards agriculture sustainability by means of soil inoculation with useful rhizospheric microorganisms promoting plant growth under various physical stresses (Kumar and Verma 2018). This group of root-linked microbes is defined as plant growth-promoting rhizobacteria (PGPR). They belong to various genera (*Bacillus*, *Pseudomonas*, *Enterobacter*, *Burkholderia, Klebsiella, Variovorax, Azospirillum*, *Azotobacter, and Serratia*) and are used to promote plant growth under both normal and stress conditions (Vimal, Singh et al. 2017).

The root rhizospheres is the most dynamic and ecological niche containing various root exudates influencing the surrounding soil microbial flora (Hartmann, Rothballer et al. 2008). Plant photosynthetic products comprising 5%–30% of the root exudates (Ali, Charles et al. 2014), and the presence of a number of low molecular weight compounds such as ions, free oxygen and carbon, mucilage, and different primary and secondary plant metabolites (Sekar and Kandavel 2010). PGPR plays a fundamental role in plant growth even under stressful conditions. They can assist in the proliferation and development of plants by either indirect or direct mechanisms. They actively take part in major plant growth through utilization of soil nutrients, control of hormones which play a crucial role in plants, adjust nutritions in plants, and help to secret plant growth supporting regulators (De Bruijn 2015, Katayama, Baba et al. 2015, Spence and Bais 2015)..

2.4 Potential use of biofertilizer for sustainable agriculture.

Biofertilizers are commonly defined as “Preparation containing live microbes which help in enhancing the soil fertility either by fixing atmospheric nitrogen, solubilizing phosphorus, and producing growth hormones with their biological activities”. The soil inoculated with useful bacteria, also known as plant growth-promoting bacteria (PGPB) consist of significant approaches toward sustainable agriculture goal due to their productive effect on plant growth and improvement via numerous mechanisms (Huang, Song et al. 2013, Gouda, Kerry et al. 2018, Sambo, Nicoletto et al. 2019). They have been promoted to yield the naturally available biological system of nutrient mobilization which

increases soil fertility and ultimately helps to obtain a high crop yield (Pandey and Singh 2012). These microorganisms play a crucial role by delivering nitrogen followed by other mechanisms, such as the production of siderophore, exopolysaccharides (EPS), and phytohormone; phosphate solubilization; and protection against phytopathogenic fungus (Figueiredo, Barroso et al. 2008, Hospido, Carballa et al. 2010, Egamberdieva 2013). Another advantageous part is that after regular use of biofertilizer for 3–4 years, the application of inoculum is not needed, as parental inoculum will be enough for growth and proliferation (Bumandalai and Tserennadmid 2019).

Biofertilizers are widely found in a diversity of habitats. They are free-living in soil and water, have symbiotic association with grasses, symbiotic association in termite guts, actinorhizal association with woody plants, have cyanobacteria symbiotic relation with different plants, and have symbiotic relation between root-nodule and legumes (Dixon and Kahn 2004). Certain types of microorganisms that are used as biofertilizers are mainly identified in the plant rhizospheres where they form direct mutualistic relation with the roots (Nie, Peng et al. 2012). Biofertilizer are catagorized as free-living bacteria *(Azotobacter and Azospirillium),* blue-green algae, and symbionts, including Rhizobium, Frankia, and Azolla. The N2-fixers bacteria are connected with legumes include *Rhizobium, Mesorhizobium, Azorhizobium, Bradyrhizobium, Sinorhizobium,* and *Allorhizobium* and those with non-legumes involve are *.Acetobacter, Azomonas, Beijerinckia, Clostridium, Bacillus, Enterobacter, Derxia, Corynebacterium, pseudomonas spp*. Etc (Meena, Mishra et al. 2017).

2.5 Morphological characters of *Azotobacter.*

Among the different nitrogen fixing bacteria, *Azotobacter* is a free-living, diazotrophic nitrogen-fixer where it performs an significant role in the nitrogen cycling because of its different metabolic properties (Sahoo, Ansari et al. 2014). *Azotobacter* spp. has several metabolic capabilities; it has the highest metabolic rate of any organisms (Curatti, Brown et al. 2005). It can well grow at a pH range of 4.8–8.5 and fixes N at optimum pH of 7.0–7.5. The

favorable temperature needed for the growth is 28–32 ° C and the maximum temperature is around 38°C and the minimum is 22 ° C. (Aquilanti, Favilli et al. 2004, Gurikar, Naik et al. 2016, Jiménez, Saldarriaga-Isaza et al. 2021). *Azotobacter* can deposit at least 10 μg of nitrogen per gram of glucose utilized.

Azotobacter species which are free living nitrogen fixers are widely connected with nitrogen fixation [9], production of different physiologically functional growth regulators like gibberellin, auxin and cytokinin (Azcón and Barea 1975, Kukreja, Suneja et al. 2004) ammonia, vitamins and growth substances that are required for seed germination (Narula, Lakshminarayana et al. 1981, Gonzalez-Lopez, Salmeron et al. 1986) protection against root pathogens (Verma, Kumar et al. 2001, Sindhu, Rakshiya et al. 2009) stimulation of beneficial rhizospheres microorganisms resulting enhancement of plant yield (Wu, Bernard et al. 2009). *Azotobacter* also produces extracellular polysaccharides such as alginates, leavens, and cellulose and to accumulate polyhydroxybutyrate (Anjum, Gill et al. 2015).

The genus *Azotobacter* belongs to the γ-subclass of the Proteobacteria and consists of eight *species A. armeniacus, A. beijerinckii, A. chroococcum, A. nigricans, A. paspali, A. salinestri, A. tropicalis, and A. vinelandii* reported by (Beesley, Moreno-Jiménez et al. 2011, Özen and Ussery 2012). A complete study of taxonomy and ecology of the *Azotobacteraceae* investigating numerous morphological, physiological and biochemical characteristics of the aerobic N2-fixers was studied by Thompson & Skerman (Aktar). The taxonomic arrangement of *Azotobacter* has been reassessed the usage of nucleotide sequence comparisons resulting to the reallocation of *Azotobacters* to the family *Pseudomonadaceae* (Mącik, Gryta et al. 2020).

Azotobacter is an aerobic, heterotrophic, and free-living N2-fixing bacteria, which can be isolated from soil, water, and sediments [2].(AZO21) These bacteria are gram-negative, non-motile, but often motile by peritrichous flagella while others may be immotile .They are mostly oval or round in shape and shaped thick-walled cysts (dormant cells resistant to deleterious conditions) under unfriendly environmental surroundings (Martyniuk and Martyniuk 2003) The cell size ranges from 2 to 10 mm long and 1 to 2 mm

wide. The genus *Azotobacter* was found in 1901 by Dutch microbiologist, botanist and initiator of ecological microbiology-Beijerinck, and is responsive to acidic pH, high-level of salt concentration and temperature (Aquilanti, Favilli et al. 2004).

2.6 Overview of *Azotobacter chrococcum* as biofertilizer

Among the different species of *Azotobacter, A. chroococcum* was the first species described (Abdelmajeed, Khelil et al. 2012). *A. chroococcum* is known to be most prevalent species found than other species including *A. agilis*, *A. vinelandii*, *A. beijerinckii*, *A. insignis*, *A. macrocytogenes* and *A. paspali (Newbould 1989)*. *A. chroococcum* is common in neutral or alkaline soils. Many studies had been in this strains have tested their capability to encourage plant proliferation either through the formation of plant growth components, mineralization or supplementation of deposit nitrogen or a combine form of these factors (Asif, Mughal et al. 2018) (Ishac, El-Haddad et al. 1986, Haahtela, Kilpi et al. 1988, Rai and Gaur 1988) reported that *A. chroococcum* inoculated to cereal plants has capable to add number of root hairs, tilling ratio, dry matter concentration, N uptake and boost yields of wheat.

Many research have proven that *A. chroococcum* as soil inoculum is not only fruitful in N fixation however it has different other attributes including generation of growth hormones (Remus, Ruppel et al. 2000) production of fungicidal substances, producing of siderophore and the property to solubilize phosphate (Kumar and Narula 1999, Narula and Dunning 2000).

In addition other study demonstrates inoculation with *A. chroococcum* brought about a appreciable growth in plant in relation to control (Romero-Perdomo, Abril et al. 2017). (Fatemeh, Masoud et al. 2014) reported that the application of PGPR involving *Azotobacter chroococcum*, combining with other strain *Azospirillum lipoferum, Pseudomonas fluorescens* and *B. subtilis* drastically stimulated percent of seed germination, pace of germination and mean germination time of Crataegus pseudoheterophylla Pojark plants. Higher percentages of seed germination (18.33%) and speed of germination (4.82 number /day) were recorded for treatment containing all bacterial inoculants.

(Ahmad, Ashfaq et al. 2016) observed that disease prevalence with the aid of using root-knot nematode Meloidogyne incognita was finely decreased when *A. chroococcum* was implemented to chickpea plants. The utilization of *Azotobacter chroococcum* in many research had found in experiments as microbial inoculum through aid of growth components and their effect on the crop plants has remarkably improved crop yields in agriculture system (Bageshwar, Srivastava et al. 2017)

2.7 Plants growth promotion activities of Azotobacter

2.7.1 Nitrogen fixation

BNF is one of the natural process to contribute atmospheric nitrogen that is converted to ammonia by free living nitrogen-fixers utilizing an enzymatic compound known as nitrogenase (Lubell, Hillis et al. 2011, Garnett, Appleby et al. 2013). Nitrogen-fixing microorganisms are described as prokaryotes (Bacteria and Achaea), comprising hundreds of species. They are divided into aerobic, micro aerobic, anaerobic, and photosynthetic bacteria, and actinomycetes (Russelle 2008). Those microorganisms which have the capability to add atmospheric nitrogen (N2) are called diazotrophs. Biological transformation of N2 to ammonia is a complicated process which is conducted by the enzyme nitrogenase, the major enzyme complex found in the microbes able to fix atmospheric Nitrogen (Rodrigues, Ladeira et al. 2018, FERREIRA, Arrobas et al. 2020).

As mention earlier biological nitrogen fixation is an energetically expensive process because 16 ATP molecules are needed to break down an N_2 molecule. Twelve additional ATP molecules are required for $NH4^+$assimilation and transport, totally 28 ATP molecules are required. The nodulating plants must provide 12 g of glucose to their bacterial partners to benefit 1 g N in part (Giri, Giang et al. 2005). Normally the Haber–Bosch process requires a temperature of 400–500 _C and a pressure of ~200–250 bars to produce the same amount of nitrogen (Gilchrist and Benjamin 2017).

(Burén and Rubio 2018, Nonaka, Yamamoto et al. 2019) reported that nitrogenase enzyme is quite similar in most of the nitrogen-fixing bacteria. It

is an enzyme complex consisting two metal components: dinitrogenase MoFe (molybdenum-iron protein) serving as the catalytic component and dinitrogenasereductase (Fe protein). These two components are encoded by the nif genes, the nifD and nifK genes coding for MoFe dinitrogenase and the nifH gene coding for Fe dinitrogenase reductase. The enzyme nitrogenase is extremely sensitive to oxygen because it is inactivated in aerobic environment. In fact, oxygen inactivates and destroys nitrogenase and has an inhibitory effect on nitrogen fixation and assimilation pathways (Berman-Frank, Chen et al. 2005).

2.7.2 Plant growth promoting hormones

Azotobacter is the most extremly studied genera in the group of heterotrophic free-living N_2-fixing bacteria because of its potentiality to fix atmospheric N, with the production of biologically active growth-promoting substances. (Thakur and Sharma 2005). Plant hormones cpmorises of organic components generate by both the microorganisms and also by plants. They show either stimulatory or inhibitory effects on many of physiological and biochemical activities in microorganisms and plants too (Ansari and Mahmood 2019). Production of plant growth regulators including phytohormone and vitamins is a basic characteristics among *Azotobacters* (Arshad and Frankenberger 1991). *Azotobacter* are found to produce physiologically active substances like vitamin B_{12} thiamine, riboflavin, pyredoxin, gibberllins, auxins (IAA), nicotinic acid, folic acid, pantothenic acid and biotin. *Azotobacter* also produces traces of indole acetic acid, folic acid and gibberllin like substances sufficient to improve plant physiology (Lakshmi-Kumari, Vijayalakshmi et al. 1972).

A. chroococcum when used as inoculum was an effective biological management option in tomato fertilization program. It has been found that IAA perform a crucial role in different cellular activity, such as cell division, differentiation, and vascular bundle formation (Spaepen, Vanderleyden et al. 2009, Babalola and Glick 2012).

2.7.3 Pesticide degradation

Microorganisms are recognised to be powerful degraders of insecticides in infected soils. Several kinds of pesticides are found to have direct effect on soil microbiology, environmental contamination, and health risks in all living nature. Additionally, microorganisms are observe in toleration and biodegradation of chemicals which adversely have an effects in the population of soil microorganisms, viz., *Azotobacter* , *Pseudomonas* , etc (Chennappa, Naik et al. 2017, Miller-Robbie, Ramaswami et al. 2017).

Azotobacter sp.is capable to utilize aromatic components has been demonstrated for many years. *Azotobacter* sp is noted to be useful in degrading the byproduct of aromatic compounds like benzoate, p-hydroxy benzoate, protocatechuic acid, 2,4-D,2,4,6- Trichlorophenol, etc.in the contaminated soils (De la Rosa, Mayol et al. 1999, Barman and Varshney 2008). Additionally, *Azotobacter* sp. had been observed to decompose wide range of many other chlorinated phenols like 2-Chlorophenol, 4-Chlorophenol, 2,6-Dichlorophenol and 2,4-6-Trichlorophenol (Wang, Gao et al. 2009). (Moneke, Okpala et al. 2010) reported the biodegradation of glyphosate herbicide artficially using *Azotobacter* isolates from rice, thus aid in natural farming system to enhance soil nutrients and fertility and also help to reduce environmentally toxic chemicals from food products.

2.7.4 Disease management

Plant disease management by using the bio products is an ecofriendly and novel procedure (Meng, Jiang et al. 2012, Akram, Rizvi et al. 2016). A greater number of plants growth promoting rhizobacteria have been observe to generate antifungal metabolites that kill some pathogenic bacteria, fungi, and viruses (Akram, Rizvi et al. 2016). Among the different group of bacteria, *Azotobacter* is also proven to suppress the pathogenic diseases of crop plants. (Chen, Suter et al. 2008) reported that along with the production of growth stimulates (IAA, gibberellins, cytokinins), *Azotobacter* maintains a healthy niche for growing plants by inhibition of phytopathogenic fungi through antifungal substances. (Maheshwari, Dubey et al. 2012) eperimented that the strain TRA2 of *A. chroococcum* regarded as an isolate of wheat rhizosphere

confirmed potent antagonistic activity against root rot fungus *Macrophomina phaseolina* and *Fusarium oxysporum*, also improved appreciable production of wheat. (Elhami, Akram et al. 2016) observed that root knot nematode *Meloidogyne incognita* effectively decreased disease incidence when *A. chroococcum* was inoculated to chickpea plants.

Apart from, *Azotobacter spp* have the capacity to provide siderophores that bind to the available form of iron Fe+3 in the rhizosphere, thereby lacking the phytopathogens from iron availability and shielding the plant health. (Jnawali, Ojha et al. 2015). Examples of the fungal pathogens which are control by the application of *Azotobacter* as a biopesticides includes *Alternaria, Fusarium, Rhizoctonia, Macrophomina, Curvularia, Helminthosporiumand Aspergillus* (Jnawali, Ojha et al. 2015).

2.8 Response of crop to *Azotobacter*

Among the free-living N2-fixing bacteria, *Azotobacter* is the well studied genera due to its capability to deposit nitrogen in atmosphere, along with the production of biologically active growth-enhancing components such as Indole acetic acid, gibberellins and B-vitamins (Thakur and Sharma 2005). In recent years, microbial inoculums containing N_2-fixing and other useful microorganisms have been experimented on potted plants, nurseries and field crops (El-Sirafy, Woodard et al. 2006, Aseri, Jain et al. 2008, Chakraborty, Nagarajan et al. 2008, Jahan 2013, Ansari, Tipre et al. 2015).

Many researches have been done to proven the effects of inoculation of cereals with *Azotobacter* spp and these studies demonstrates that *Azotobacter spp* have increased grain yield and N concentration in plant (de Freitas 2000, Narula, Kumar et al. 2001, Emtiazi, Ethemadifar et al. 2004). *Azotobacter* has been used as a bio fertilizer for all non-leguminous plants, mainly for rice, wheat, cotton, vegetables, sugarcane, sweet potato, tomato and sweet sorghum. The study done by (Dutta and Singh 2002) reported a significant increase in seed yield in rapeseed and mustard when *Azotobacter* is used as inoculum. It fixes almost 30kg N per year. It is mainly commercialized for

sugarcane crop, as it helps to grow the cane yield by 25–50 tons/hectares and sugar content by 10–15% (Kizilkaya 2009).

2.9 Tomato

Solanum lycopersicum L. is a tomato plant, one of the most widespread horticultural crops worldwide and is ranked second next to potato (Pastor, Carlier et al. 2012). Approaches for growing fruit production and tomato quality are therefore, of wide interest to producers (Flores, Sanchez-Bel et al. 2010) due to its major role in health of human beings (Chapagain and Wiesman 2004). World production for processing tomato was estimated at 34.8 million tons in 2018 (Guerrieri, Fanfoni et al. 2020).

Tomato is mainly cultivated during winter and summer seasons. It grows mostly under an average monthly temperature range of 21˚C–23˚C. The best soil for cultivating tomato is a fertile loam soil with more sand in the surface layer, and clay in the sub-surface layers. Tomato can grow well having soil pH 6.0–7.0 (Verma, Kumar et al. 2009). Tomato is the important source of vitamin C and vitamin A (Tyssandier, Feillet-Coudray et al. 2004), lycopene (carotenoids), pro-vitamin A, β-carotene and flavonoids (Friedman 2013). Since, tomato need greater quantity of NPK for its growth and development, the absence of any one of these nourishing components limit its growth and production poorly. In relation to horticultural crops, tomato production is also determined by PGPR application (Ibiene, Agogbua et al. 2012). *Azotobacter* and *Azospirillum* when utilize both, alone or as mixture inoculum, notably enlarged the total plant dry weight, plant height, number of leaves per plant, number of fruits per plant, yield per plant, average fruit weight per plant, chlorophyll and protein content of tomato plants (Ordookhani, Khavazi et al. 2010, Ramakrishnan and Selvakumar 2012).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipment, media and chemical reagents used in this study are listed in Appendix A

3.2 Methods

3.2.1 Study design

This study was conducted from December 2020 to June 2021. The research work was carried out in microbiology laboratory of Central Campus of Technology, Dharan.

3.2.2 Soil sample collection

The 25 soil samples (each bag 100g) were collected randomly from different rhizospheric areas of forest surrounding of Panbaari Dharan. Soil samples were withdrawn at a depth of 10–15 cm below the surface and collected into sterile polythene bags with labeling and immediately brought to the laboratory. These samples were preserved in laboratory refrigerator at 4°C temperature for further study.

3.2.3 Cleaning and sterilizations of glassware

The different glassware such as petriplates, pipettes, beakers, conical flasks, test tubes *etc*. used in this study were sterilized in hot air oven at 160°C temperature for at least 2 hours. Likewise, water, medium was sterilized by autoclaving at 15 pounds per square inch pressure for 30 minutes

3.2.4 Analysis of physical properties

3.2.4.1 Moisture content (MC):

Moisture content (MC) was calculated using the gravimetric method. For the determination of moisture content 20 g of soil was taken in pre-weighted petridish and then oven-dried at 105 °C to constant weight for about 24hrs and

the dry weight was recorded (Yerima and Van Ranst 2005). These values were then used to determine the moisture contents of the soils using the formula:

$$MC\% = \frac{Fresh\ weight\ of\ soil - dry\ weight\ of\ soil}{dry\ weight\ of\ soil} X100\%$$

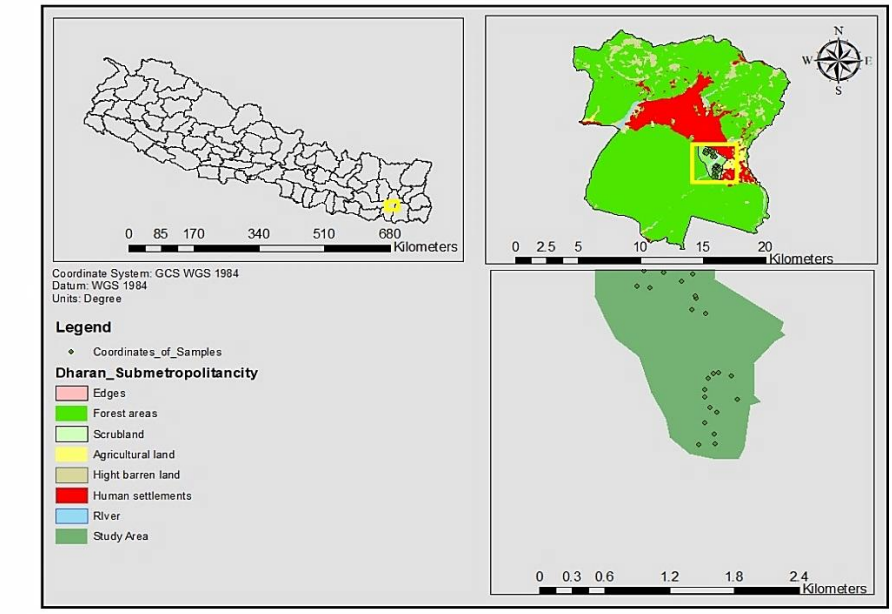


Figure 1:Site Map of Soil Sample Collection in Panbari, Dharan.

3.2.4.2 Water holding capacity (WHC):

About 10 ml of water was uniformly placed in a filter paper placed in a funnel. The oven dried and crushed soil sample (about 20g) was put into the funnel. After that water was poured uniformly with the help of glass rod until a drop of water was seen passing from the tip of funnel. The volume of water retained by the dry soil sample was noted (Yerima and Van Ranst 2005). And the water holding capacity of soil sample was calculated.

$$water\ holding\ capacity = \frac{volume\ of\ water\ retained}{mass\ of\ dry\ soil} \times 100$$

3.2.4.3 Soil pH:

PH was measured in water at ratio 1:5 (soil: water) by glass electrode pH meter. About 20g of soil taken and 100ml of distilled water is added and stirred for 10 minutes. The mixture was then allowed to settle down for 30

minutes and the pH was measured using calibrated electronic pH meter (Yerima and Van Ranst 2005).

3.2.5 Analysis of chemical properties

3.2.5.1 Soil carbon:

Estimation of soil organic carbon was done by Walkley-Black chromic acid wet oxidation method (D L Heanes 1984). The soil sample was dried, grinded and sieved through 2mm sieve. It was again sieved through 0.5mm sieve and 0.5 g sample was taken in 250ml conical flask. 10ml of 1N potassium dichromate was added to the sample and then 20ml of conc. H_2SO_4 was added with swirling to disperse the soil. A 200℃ graduated thermometer was inserted inside the flask and heated in gas burner over asbestos gauze. When the temperature was 135℃, the flask was kept aside to cool slowly. After 30 minutes, 200ml of deionized water was added and it was titrated with $FeSO_4$ using ferroin indicator. Blank titration was performed in similar manner without sample. The organic carbon content was calculated using given formula:

$$\text{Organic carbon \%} = 0.003 \times N \times 10 \times 1\text{-}TS \times 100/ODW$$

Where, ODW = oven dried weight

N = Normality of $K_2Cr_2O_7$

T = Volume of $FeSO_4$ used in sample titration (ml)

S = volume of $FeSO_4$ used in blank titration (ml)

3.2.5.2 Soil potassium:

Soil potassium was estimated by following protocol: P05-001A. The air-dried soil sample was passed through 2mm sieve. 10gm of sieved soil was transferred to a 100ml volumetric flask together with 50ml of the ammonium acetate/ acetic acid solution. The flask was transferred to a shaker and the sample solution was shaken for 30 minutes. The flask was removed from the shaker, allowed to stand for several minutes and then decanted the supernatant liquid through a dry Whatmann no. 2 filter paper. Potassium standard solution was prepared using potassium chloride and ammonium acetate/acetic acid solution. And then, standard solutions of 20, 40, 60, 80 and 100 ppm were

prepared. Calibration of flame photometer was done by aspirating these five-potassium standard solutions. After calibration, potassium content of sample was estimated by aspirating the sample solution.

3.2.5.3 Soil nitrogen:

Soil nitrogen (N) was determined using the Kjeldahl distillation method where 2 gram of soil was placed in 250ml digestion flask followed by the addition of 2 gram of catalyst mixture and 25ml of conc. H_2SO_4. Blank sample was prepared likewise without soil sample. The sample and the blank were allowed to digest till green color is seen. The digest was then allowed to cool followed by addition of 20 ml of distilled water. After ensuring settlement, the supernatant solutions were decanted into 100 ml volumetric flasks. 5 ml of sample and blank was taken one at a time into distillation assembly with 10 ml of 30% NaOH and the solution was distilled for 5minutes. The released ammonia was trapped in 5ml of 2% Boric acid containing 4 drops of mixed indicator. The boric acid mixture in the flask was titrated with standard 0.01N HCl until color disappear (Yerima and Van Ranst 2005). The nitrogen content was then calculated by using formula:

$$N(\%\ wet\ basis) = \frac{(Sample\ titer - blank\ titer)X\ No\ of\ HCL\ X\ 14\ X100X100}{aliquot(ml)X\ weight\ of\ sample(gm)X\ 1000}$$

3.2.6 Isolation of *Azotobacter* species

Soil samples were collected from 10cm to 15cm and taken to the laboratory in sterile polyethylene bags. For microbiological analysis, for the isolation of *Azotobacter spp*, 1gm of soil sample was measured and added to 10 ml of sterile water making dilution of 10^{-1} and serially diluted up to 10^{-5}. From these different dilution factor i.e 10^{-3} and 10^{-4}, 0.1 ml suspension was spread onto the nitrogen free Ashby's glucose agar plates with the help of L- shaped dolly rod and incubated at 30˚C for five days. After 5 days of incubation, the isolated colonies were observed. Colonies which show *Azotobacter* like colonies on Ashby's medium were selected for screening of *A. chroococcum* and purified by sub culturing onto Ashby's media and preserved at 4˚C for further identification (Upadhyay, Kumar et al. 2015).

3.2.7 Identification of *Azotobacter chroococcum*

The obtained isolates were characterized on the basis of colonial characteristics, microscopic examination, pigmentation and biochemical tests as described in Bergey's Manual of Systematic Bacteriology. Colonial characteristics were noted by the appearance of well-developed colonies after incubation period. Gram staining was performed. The different biochemical tests include motility, catalase, mannitol, maltose, glucose, sucrose and starch hydrolysis were performed to identify as *Azotobacter chroococcum* (Kennedy, Rudnick et al. 2015).

3.2.8 Pot experiment

The nitrogen fixation evaluation of *A. chroococcum* in tomato plants was studied in pot experiment in home garden at normal condition at 26˚C.

3.2.8.1 Tomato seedlings preparation

Tomato seedlings were prepared before potting. The seeds were surface sterilized with 70% ethanol for 2-3 min, followed by 10 min in mercuric chloride solution and were finally washed with autoclaved distilled water to remove the traces of treated chemicals. And then, seeds were sown in plastic trays containing autoclaved field soil for 25 days until the seedlings were ready for planting (Ahmed, Syed et al. 2021).

3.2.8.2 Inoculum preparation

Before inoculation of tomato seedlings, freshly grown bacterial culture of *A. chroococcum* was added to 10ml Nutrient broth with a sterile loop and allowed to grow for 24 hours at 30˚C. After 24 hours, bacterial culture was transferred to 100 ml conical flask containing NB with bacterial cell 10^7 cells/ml and incubated for five days at 30˚C. Then, the broth in conical flask was kept in rotatory shaker for two hours to mix all bacterial cells uniformly (Ahmed, Syed et al. 2021).

3.2.8.3 Potting

The experiment was carried out in the clay pots of 5kg capacity. For pot filling, field soil was used and it was mixed physically to make homogenous soil. The physical properties (pH, moisture, water holding capacity), and

chemical properties nitrogen, potassium and carbon of soil were studied. The soil was sterilized by autoclaving for 2 hours two times and filled in clay pots.

The 30 days old healthy tomato seedlings were prepared for root inoculation. The tomato seedlings were picked and made soil free by washing with sterile water for 5-6 times. And the seedlings were dipped into the nutrient broth containing bacterial suspension for half an hour before transplantation to the pots (Singh, Phukan et al. 2018).

The seedlings were transplanted in twelve pots containing four treatments with three replications. The experimental treatments included sterile soil with *A. chroococcum* non inoculated(S-NI), non- sterile soil with *A. chroococcum* non-inoculated (NS-NI), sterile soil with *A. chroococcum* inoculated(S-I), non- sterile soil with *A. chroococcum* inoculated (NS-I) (Wang, Wang et al. 2020).

Plants were maintained in normal conditions at garden by watering daily and equal moisture was maintained in each pot. In all treatments, plant height, number of leaves were measured at 20, 40, 60 days after transplantation. The root length was measured after 60 days. And the nitrogen content of soil from all treatments was analyzed.

3.2.8.4 Analysis of parameters

a) The height of the plant (cm):

The height of tomato plant was measured from the base to the tip of the tallest tiller of the plant. It was straightened up, measured with a ruler placed alongside and its height is noted.

b) Number of leaves:

Total number of leaves per plant was counted and recorded.

c) The root length of the plant (cm):

It was measured from lower to upper rhizospheres region with scale after harvesting.

d) Evaluation of nitrogen content of soil:

Soil nitrogen (N) of twelve pots soil were determined using the Kjeldahl distillation method after harvesting of tomato plants as described in **3.2.5.3**

3.2.9 Data Analysis:

The data recorded from pot culture was documented and tabulated. One-way ANOVA test was used to determine the association of plant growth parameters with different treatments. Using JMP version 14 furthermore Turkey's honesty test was done to find the significant relationship between the treatments and days at $p<0.05$. Graphical interpretation was done with the help of ggplot2 package in R programming version 4.02.

3.2.10 Methodology Design

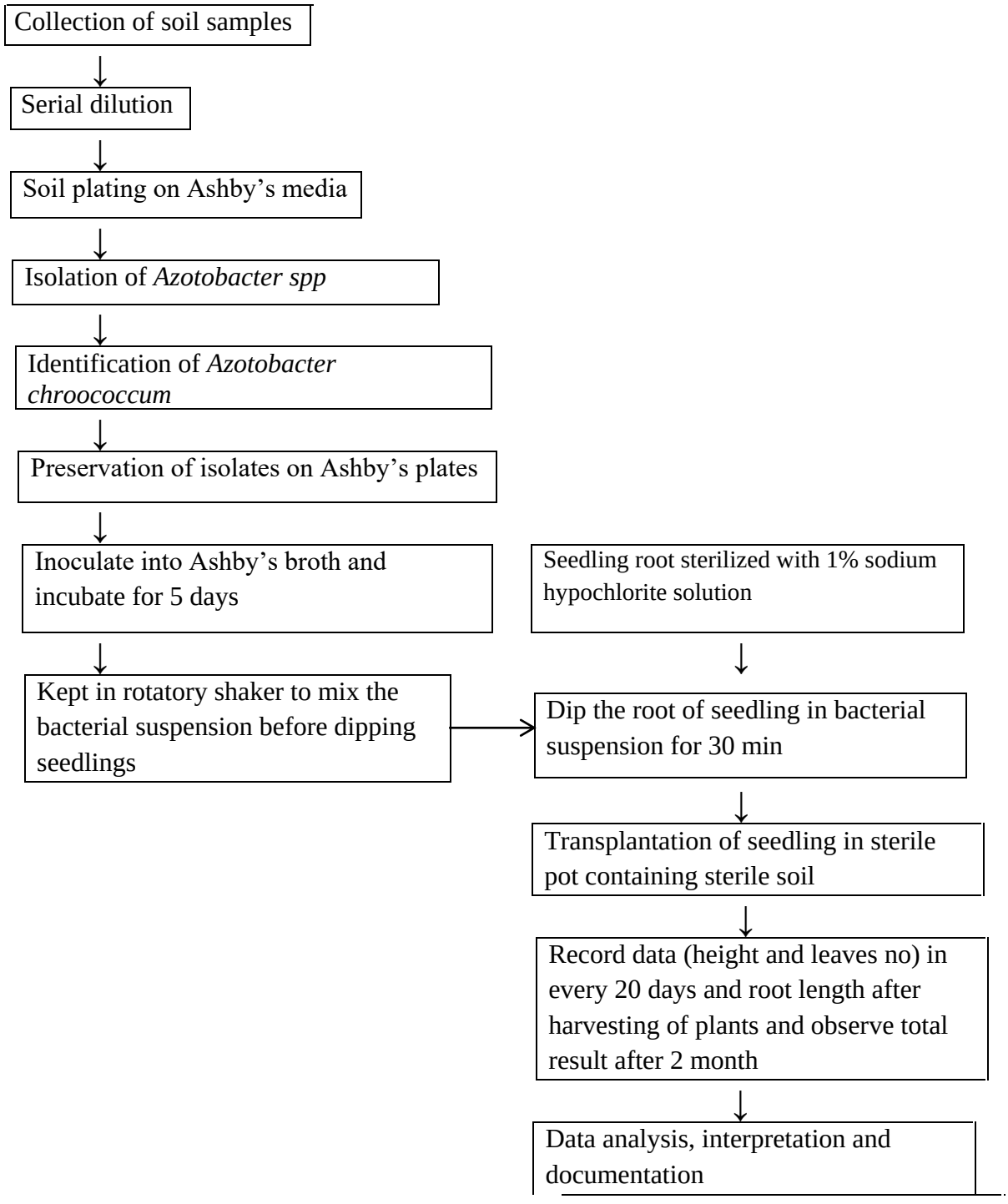


Figure 2: Schematic diagram for characterization of Azotobacter chroococcum and its effects on growth of tomato plants.

CHAPTER IV

RESULT

4.1 Physical properties of soil

The soil samples were collected from different area of forest surrounding of Panbari Dharan. The physical properties of 25 soil samples were studied which are listed in table no.**1** The soil moisture content ranged from 2.81% to 15.01% for all soil samples with an average value of 8.46%. The value of moisture content differs mainly depending upon the type and texture of the soil. The water holding capacity of soil samples ranged from 15.5% to 56% with an average value of 47.18%. The pH value of soil samples were acidic ranging from 4.4 to 6.5 with an average value of (5.5). The nature of collected soil samples around the forest surrounding were mainly loamy sand.

Table 1: Physical properties of soil

SN	Soil temperature	Moisture content (%)	Water holding capacity (%)	pH
1	15.3˚C	14.32	52.5	5.2
2	17.3˚C	11.01	55	4.4
3	16.9˚C	15.01	56	5.2
4	16.8˚C	6.49	54	4.7
5	15.9˚C	12.22	51	5
6	16.2˚C	12.53	55	4.9
7	16.6˚C	11.89	56	5
8	16.3˚C	13.16	55	5.2
9	17.1˚C	11.16	5	5.4
10	17.1˚C	11.55	54	5.3
11	16.9˚C	11.12	47.5	5.6
12	19˚C	2.99	46	5.8
13	15.5˚C	6.76	49	5.4
14	16.1˚C	6.67	51	5.3
15	20.1˚C	4.73	47	5.5
16	17.3˚C	5.18	48	5.4
17	16.9˚C	3.48	37.5	5.7
18	20.2˚C	5.53	45	4.8
19	17.9˚C	2.81	38	5.9
20	17.0˚C	3.92	3	6
21	19˚C	5.89	44	5.8
22	16.3˚C	5.65	38.5	6
23	16.9˚C	14.86	15.5	6
24	16.8˚C	9.05	52	6.1
25	18.9˚C	3.53	39	6.5

4.2 Chemical properties of soil

Nitrogen content of 25 collected soil samples were studied. The soil nitrogen of studied soil samples ranged from 0.03% to 0.21% with an average value of 0.1024%.

Table 2: Nitrogen content (%) of soil

Sample no.	Nitrogen (%)
1.	0.13
2.	0.13
3.	0.10
4.	0.10
5.	0.10
6.	0.11
7.	0.18
8.	0.14
9.	0.07
10.	0.03
11.	0.14
12.	0.08
13.	0.09
14.	0.21
15.	0.04
16.	0.07
17.	0.04
18.	0.13
19.	0.07
20.	0.09
21.	0.07
22.	0.11
23.	0.15
24.	0.10
25.	0.08

4.3 Isolation and Identification of *Azotobacter chroococcum*

From this study, twenty-five soil samples were collected, 36 isolates were selected as *Azotobacter spp* based on coloration, colony morphology and microscopic examination. The colonies were found to be milky white, flat, circular, creamy, convex and glistening at early growth, and show variation in colour at ageing colonies depending upon different species of *Azotobacter*. The isolates were studied microscopically, Gram staining showed gram negative oval rod, small rod, coccoid and ellipsoidal cell shape.

Of these 36 isolates, further biochemical tests were done. All isolates were catalase positive, Since *Azotobacter spp* show catalase positive. Most of the species of *Azotobacter* are motile. Here, in this study 33 isolates were motile. Among 36 isolates on the basis of utilization of carbon sources such as glucose, maltose, mannitol and starch hydrolysis test, 27 isolates showed mannitol positive, 32 isolates showed maltose positive, 25 isolates showed glucose positive and 33 isolates showed starch hydrolysis positive.

While studying their biochemical characteristics only 16 isolates were matched with *A. chroococcum,* 7 isolates matched with *Azotobacter vinelandii* and 13 isolates matched with *Azotobacter armeniacus* according to the bergy's manual of systematic bacteriology. The striking feature of *A.chroococcum* out of these three species is the production of pigments at ageing colonies. *Azotobacter vinelandii* showed yellow colony whereas both *A. chroococcum* and *Azotobacter armeniacus* showed brown colonies. At ageing colonies, only *A. chroococcum* appear in brown black pigment, and *Azotobacter armeniacus* usually change brown into red violet pigment in further growth in media. In this study, only 16 isolates showed brown black colonies. Therefore, 16 isolates showed character of *A. chroococcum* species based upon colonial characteristics, microscopic examination and biochemical tests among 36 colonies. The isolated colonies were picked and re-streaked on the NA medium to obtain pure cultures.

The isolated bacterial strain was maintained on the NA medium until further use. Morphology, Microscopic and Biochemical characteristics of *Azotobacter chroococcum* is given below in Table no.**2**

Table 3: Morphology, Microscopic and Biochemical characteristics of Azotobacter chroococcum

Characteristics	***Azotobacter chroococcum***
Gram staining	Gram Negative
Shape	Oval rod in chain and in clustered
Color	Milky white, glistening at early growth and light to dark brown on aging colonies
Consistency	Transparent, smooth, viscous moist colonies
Margin	Circular, raised, convex
Motility	+
Black brown pigmentation	+
Starch hydrolysis	+
Catalase	+
Mannitol	+
Maltose	+
Glucose	+
Urease	+

4.4 Nitrogen fixing efficacy of *A.chroococcum*

The isolates of *A. chroococcum* were inoculated to tomato plants to observe the PGPR effect and nitrogen fixation evaluation. Isolate of *A. chroococcum* was selected on the basis of microscopic examination, colony morphology and biochemical tests. Height and no. of leaves were measured at 20 days interval for 60 days after the plantation of tomato seedlings in the pots. And root length was measured after the harvest of plants on 60 days of inoculation. Four treatments were used, sterile and non-inoculated (S-NI), non-sterile and non-inoculated (NS-NI), sterile and inoculated (S-I), non-sterile and inoculated (NS-I). Each treatment was maintained in three replicates.

The obtained data were compared between inoculated and non-inoculated (control) tomato plants. The measured parameters related to plants growth were seen higher in *A. chroococcum* inoculated plants than non-inoculated plants.

4.4.1 Effect of *Azotobacter Chroococcum* isolates on height of plant.

After 20 DAT of seedlings to the pot, plant height (cm) was recorded at the interval of 20, 40, and 60 days. All the treatments of *Azotobacter chroococcum* inoculated tomato plants (NS-I, S-I) resulted in better plant height as compared to non-inoculated tomato plants (NS-NI, S-NI). As seen in Fig.3, the height length of tomato plants showed progressive growth in treatment NS-I than in treatment NS-NI in similar condition of soil i.e. (non-sterile). Similarly, in the same sterile soil, treatment S-I had shown finer result than the treatment S-NI. This may be because of producing growth promoting substances and as well as due to the fixation of nitrogen that supports plant growth by inoculated bacteria in the soil. Between these four treatments, treatment NS-I had made better improvement than other three treatments suggesting inoculum with this bacteria to the non-sterile (normal) soil may be beneficial for the growth of tomato plants.

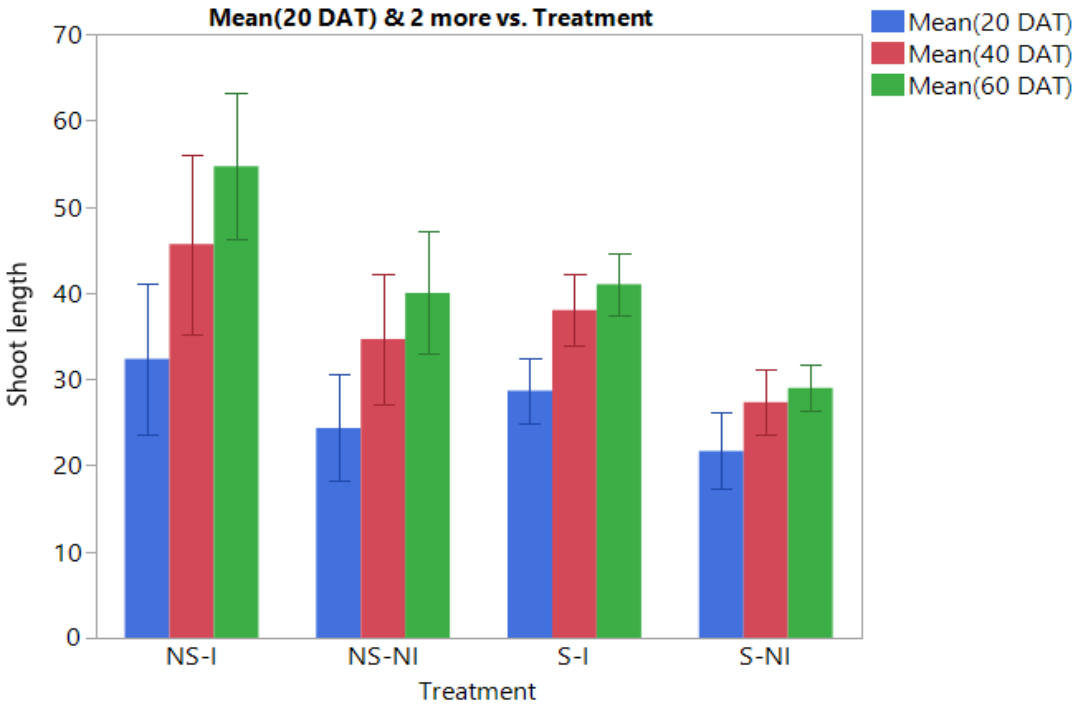


Figure 3: Effect of A. chroococcum isolates on height of tomato plant.

In each column of different colour denotes significant differences ($P<0.05$) among the treatments according to Turkey's honesty test, DAT= Days after transplantation. NS-I= Non Sterile-Inoculated, NS-NI= Non sterile-Non Inoculated, S-I= Sterile-Inoculated, S-NI= Sterile- Non Inoculated.

4.4.2 Effect of *A. chroococcum* isolates on number of leaves of plant.

After 20 DAT of seedlings to the pot, the number of leaves of tomato plant was recorded at the interval of 20, 40, and 60 days. There was difference in number of leaves in plants between the four different treatments as seen in Fig.4. The highest number of leaves were found in treatment NS-I treated with *A.chroococcum*, whereas the least number of leaves were found in treatment S-NI. The treatment NS-NI had shown greater number of leaves at 20 days, but the number decreased at 40 days, it may be due to the diseases occurrence in tomato plant, however the number of leaves had grown gradually in 60 days. Among these four treatments, tomato plants treated with inoculated bacteria in non-sterile soil (i.e. NS-I) significantly improve better growth that shows positive effect of bacteria as inoculum in the plants.

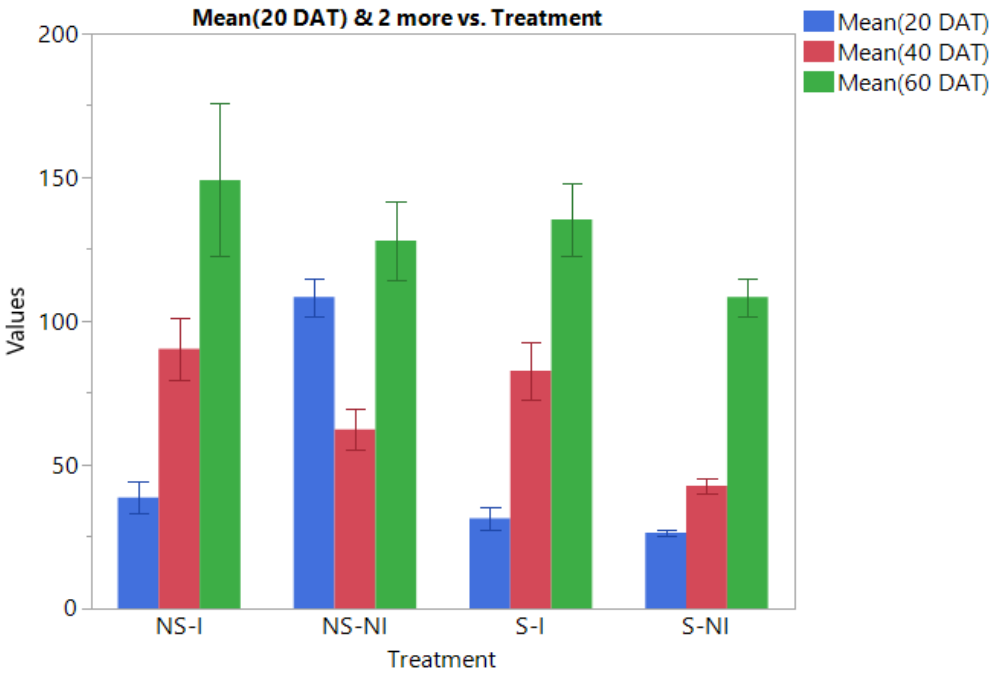


Figure 4: Effect of A. chrococcum isolates on number of leaves of plant.

In each column with different colour denote significant differences ($P<0.05$) among the treatments according to Turkey's honesty test, DAT= Days after transplantation. NS-I= Non sterile-Inoculated, NS-NI= Non sterile-Non Inoculated, S-I= Sterile-Inoculated, S-NI= Sterile- Non Inoculated.

4.4.3 Effect of *A. chroococcum* isolates on root length and nitrogen content of soil.

After harvesting the plant, the root length of tomato plants was measured at 60 DAT. As shown in the Fig 5. *A. chroococcum* significantly increases the root length. The highest root length was measured in treatment NS-I and the least was measured in treatment NS-NI. Likewise, after harvesting of plants, the nitrogen content of 12 pots soil was estimated. It was found that soil nitrogen was greater in the treatment NS-I, whereas the less nitrogen content was in the treatment S-NI. This experiment showed that non- sterile (normal) soil treated with *A. chroococcum* (NS-I) had enhanced the growth of tomato plants as well as increased the nitrogen content of soil among other treatments. The reason could be the production of growth promoting substances as well as fixation of atmospheric nitrogen to the soil which helps to increase root length and nitrogen content of soil. This study showed that *A. chroococcum* as bioinoculant can give beneficial effect on tomato plants.

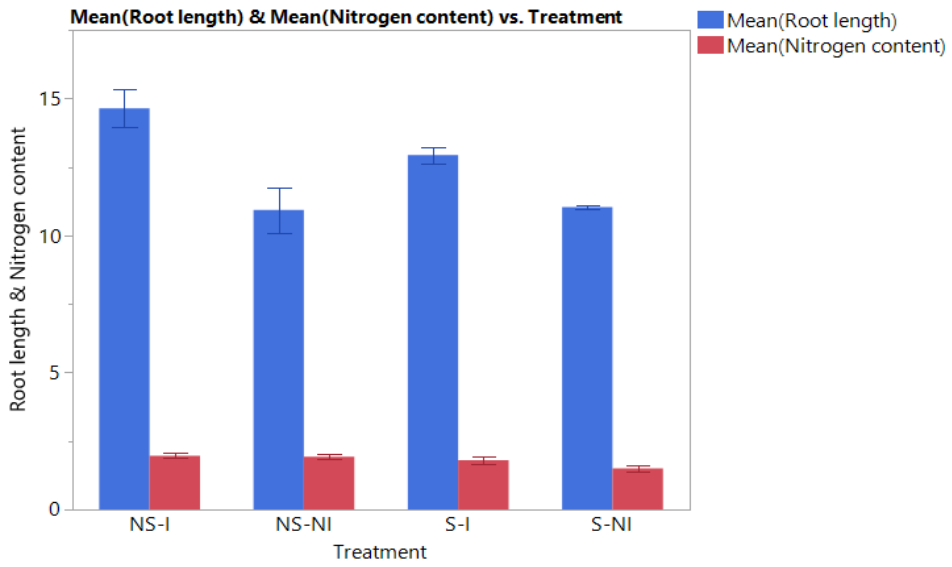
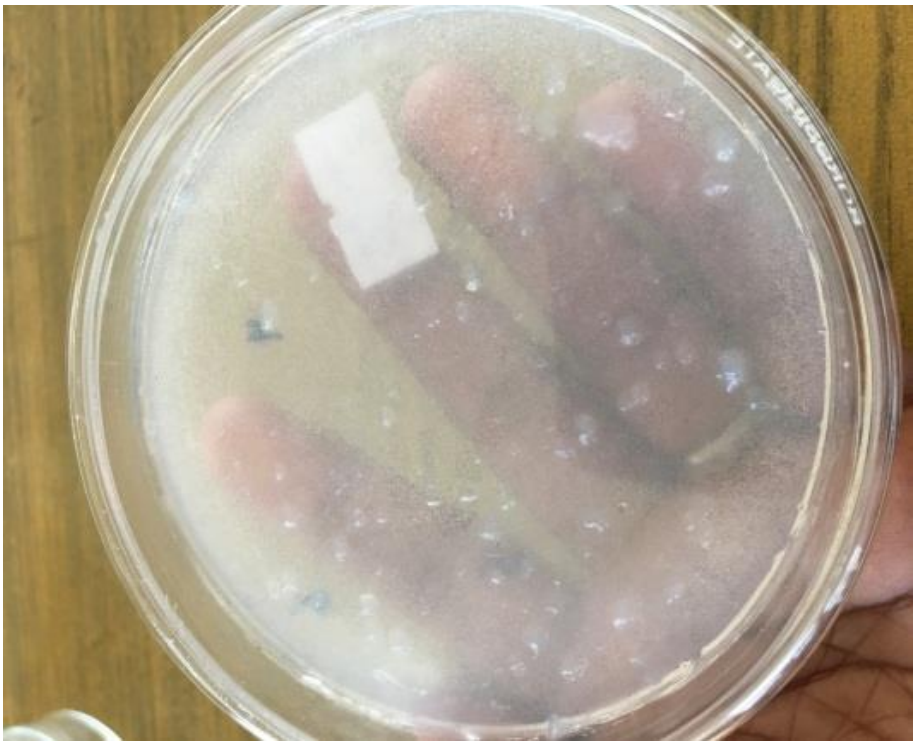


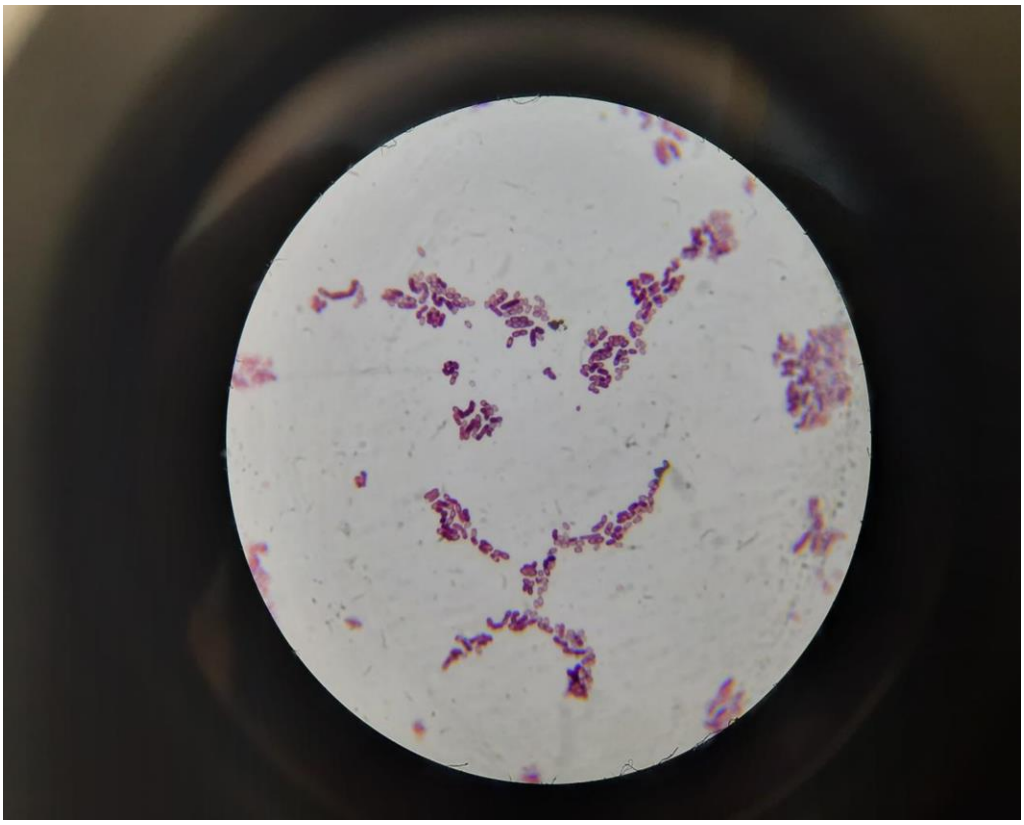
Figure 5: Effect of A. chrococcum isolates on root length and nitrogen content of soil.

In each column of different colour denotes significant differences ($P<0.05$) among the treatments according to Turkey's honesty test, DAT= Days after transplantation. NS-I= Non sterile-Inoculated, NS-NI= Non sterile-Non Inoculated, S-I= Sterile-Inoculated, S-NI= Sterile- Non Inoculated.

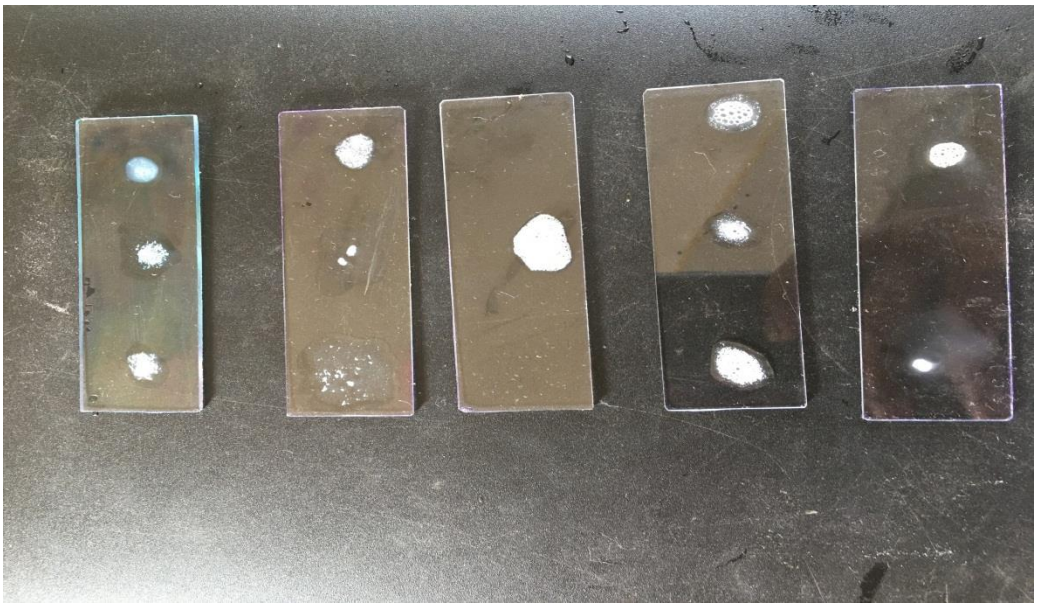


Photograph 1: Morphological view of *Azotobacter chrococcum* in Ashby's agar

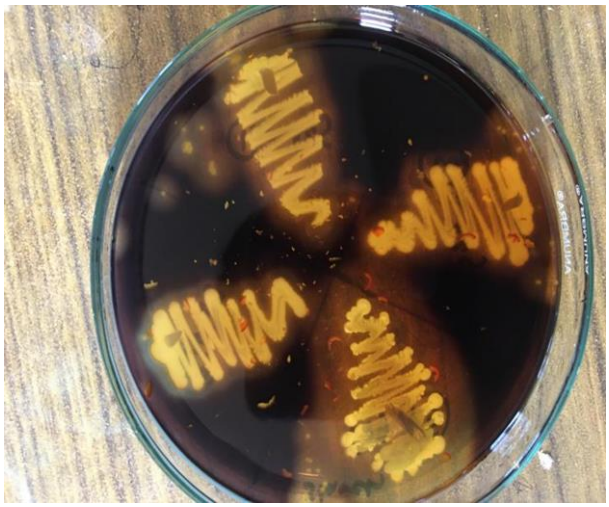
Photograph 2: Morphological view of *Azotobacter chrococcum* in Jensen's agar



Photograph 3: Microscopic view of *Azotobacter chroococcum*



Photograph 4: Catalase test



Starch hydrolysis test

Carbohydrate fermentation test

Photograph 5: Biochemical tests of *Azotobacter chroococcum*

Photograph 6: Researcher working on lab

Photograph 7: Pot experiment of tomato plants with different treatment A(S-NI), B(NS-NI), C(S-I) and D(NS-I)

Photograph 8: Pot experiment of tomato plants with different treatment A(S-NI), B(NS-NI), C(S-I) and D(NS-I)

CHAPTER V

DISCUSSION

With the extensive need of growing long-term agriculture system to feed the developing global population, agricultural bio inputs which includes PGPB inoculants have emerged as crucial equipment to decrease the usage of chemical fertilizers which have negative effect on plants and soil (Sprent and Sprent 1990). Considering all the adverse effects of continuous use of chemical fertilizers to our health, organic farming such as using biofertilizer has been developed as a potent alternative method in terms of the growing demand of healthy food supply, long-term sustainability, and concerns regarding environmental pollution (Patil, Kounaina et al. 2021). *Azotobacter* is free living nitrogen fixing bacteria which perform a vital role in managing soil fertility through numerous useful results in the rhizospheres of cereals and grasses. Beside these biological nitrogen fixation, production of growth hormones (IAA, gibberellins, cytokinins), supression of phytopathogenic fungi by antifungal components, *Azotobacter* provide a healthy niche for increasing plants (Chen, Suter et al. 2008)

According to (Dorais, Ehret et al. 2008) tomato is the second-most main vegetable in the world with a worldwide producing of 129 million tons in 2008 (Alexandratos 1995). Many approaches have been evolved for growing fruit production and better quality of tomato are hence, of key importance to producers (de Faccio Carvalho, Anghinoni et al. 2010) because of its greater role in human health. Since, tomato needs large quantity of fertilizers, the absence of any one of required nutrients could yield lower amount of tomato. Many researchers are trying to use biofertilizer to produce quality of tomato for the benefit of human health.(Aubán, Barton et al. 2015) have experimented that inoculation of tomato seedling with *Burkholderia tropica* brought about positive root proliferation of crops which further enhance to aerial tissues. Additionally, the remarkable colonization led to a sustained growth in tomato yields in two different crop seasons.(Walpola and Yoon 2013) reported that the PGPR found in tomato rhizospheres improves the better growth and build

up better shoot length, root length, fresh weight, dry weight and Phosphorus content of tomato plants.

In this study, nitrogen-fixing bacteria from soil were characterized according to Bergey's Manual of Systematic Bacteriology. And root inoculation was done to observe PGPR effects and discussed here with appropriate facts and comparison with previous works mention in the chapter.

The 25 soil samples were collected by random sampling method from forest surrounding of Panbari Dharan. Out of 25 soil samples, 36 isolates were obtained. 16 isolates were classified as *Azotobacter chroococcum*. The colonies formed by these bacteria on Ashby's mannitol agar were slightly viscous, semi-transparent, milky, circular and glistening during the early growth and later light and dark brown pigment was formed. These cultural characteristics was also described by (Upadhyay, Kumar et al. 2015) and (Abdel-Hamid, Elbaz et al. 2010) . Bacteria were Gram-negative oval rod shown in table no 4.2. Biochemical and morphological characteristics of these bacteria comprised of the following: motile, catalase and starch hydrolysis positive. Utilization of carbon source such as mannitol, maltose, glucose and utilization of urea is positive which is shown in appendix D. This finding was also reported by (Tejera, Lluch et al. 2005) and (Upadhyay, Kumar et al. 2015) for the isolation and identification of *Azotobacter chroococcum* and compared with Bergey's Manual of Systematic Bacteriology.

In this experiment, effect of *Azotobacter chroococcum* (S25Aa) on tomato plants had been observed by root dipping method (Esitken, Yildiz et al. 2010). Each tomato plant was set up with four treatments: sterile and non-inoculated (S-NI), non-sterile and non-inoculated (NS-NI), sterile and inoculated (S-I), non-sterile and inoculated (NS-I), this type of soil treatment was performed by (Wang 2020) for pot experiment. Based on the result obtained, *Azotobacter chrococcum* inoculated in tomato plants has significance difference in number of leaves and root length between the treated plants and in different interval of days. Whereas, no significance different in height of tomato plants and nitrogen content in soil between the treated plants and in different interval of

days. Our results revealed that *Azotobacter* inoculation had significant effect on growth parameters of tomato plants.

This experiment showed that non- sterile (normal) soil treated with *A. chroococcum* (NS-I) had enhanced the growth of tomato plants as well as increased the nitrogen content of soil among other treatments. Similar results was found by (Kargar, Nejad et al. 2014) when *A.chroococcum* was applied to normal soil with manure. It is also reported by (Shirinbayan, Khosravi et al. 2019) agreed that *Azotobacter chroococcum* has significant effect in maize plants. Data on the effects of *Azotobacter chroococcum* application on tomato plants are limited. (Mayak, Tirosh et al. 2004) However, many studies have been done to observe effect of *Azotobacter chrococcum* on different crops like wheat (Kargar, Nejad et al. 2014), maize (Song, Li et al. 2021), rice (Chen, Tsai et al. 2018) and vegetable crops (Rodelas, Gonzalez-Lopez et al. 1999). (Hashemi, Farnia et al. 2014) reported that when *Azotobacter* and *Azospirillum* used both, singly and as a mixture, effectively rise in the plant dry weight, plant height, number of leaves per plant, yield per plant, and average fruit weight per plant, chlorophyll and protein content of tomato plants.

In this study, four treatments were used, sterile and non-inoculated (S-NI), non-sterile and non-inoculated (NS-NI), sterile and inoculated (S-I), non-sterile and inoculated (NS-I) (Wang, Wang et al. 2020) Among four treatments, in 60 DAT the treatment (NS-I) has highest data(mean) in height(54.6), no. of leaves(149), root(14.63) and nitrogen content(1.96) whereas the treatment (S-NI) has lowest data(mean) in height(29), no. of leaves(108), root(11) and nitrogen content(1.50). There is difference in growth parameters of tomato plants between (S-NI) and (NS-I).

This experiment reveals that *A.chroococcum* when applied to normal field soil enhances the soil properties providing essential nutrients for the growth and improvement of the plants which is supported by (Mahato and Kafle 2018) when they used different forms of soil with inoculation and without inoculation. Since in the treatment (NS-I), non-sterile(normal) soil has already no. of different other microorganisms and when *Azotobacter* inoculum was added, the inoculated bacteria combining with other organisms enhanced the

better growth of height, root, number of leaves of tomato plants by fixing nitrogen to the soils and producing different growth hormones. Whereas, the treatment (S-NI) which is sterile soil with non-inoculum, due to the removal of present organisms by autoclaving soil and in the absence of inoculum, the tomato plant had no better growth. It can be clearly suggested that *Azotobacter* inoculum has positive effect in tomato plants than in the non-inoculated plants. This result is supported by (Mahato and Kafle 2018) who found similar result when *Azotobacter chroococcum* inoculum added to wheat plants and has significance effect.

(Van Oosten, Di Stasio et al. 2018) has observed that in tomato, *A.chroococcum* 76A acted as a preferred growth provider and their outcomes had proven that plants grown at sub-optimal nutritional stages and in the presence of *A.chroococcum* 76A showed better results than any other inoculums in case of shoot fresh weight, root dry weight and fruit dry weight. The impacts of *A. chroococcum* on plant yields may be related with the production of auxins, cytokinins, and GA like molecules that includes well established role in plant growth management.

In this study, inoculated bacteria have generally increased nitrogen in the pot soil. However, these increases were not statistically significant. Similar findings was stated by (Kızılkaya 2008). Since, nitrogen plays a functional role in the growth and development of the plants. This experiment showed that tomato plants inoculated with bacteria has resulted better growth in height, number of leaves and root length. This fact can explain fixation of nitrogen in the soil by inoculated bacteria might be uptake by the plants for their growth, due to the reason, less nitrogen content has seen in the soil which results there is no significant difference between the treatments. This result is supported by (Madhuri and Pandey 2008) stated that *Azotobacter* is able to convert atmospheric nitrogen to ammonia, which in turn is taken up and utilized by the plants. Same result is concluded by (Alizadeh and Parsaeimehr 2011) that the use of native, nitrogen-fixing bacteria can result in nitrogen fixation and further increase in plant yield.

Thus, *A.chroococcum* treated plants demonstrated higher values than non-inoculated controls, suggesting a protective effect of *A. chroococcum* treatment on the agriculture system.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Based on the result obtained in this study, It is observed that *A. chroococcum* has significance effect in growth of tomato plants. The treated tomato plants in normal soil have shown positive effect of bacteria (*A.chroococcum*) in the plants among the four treatments. From this study, it can be put forward that *A. chroococcum* can serve as good option as plant growth promoter for the growth of various crops in the fields in a sustainable way. The addition of this PGPR may be ideal for low-input agricultural systems where greater amounts of synthetic fertilizers may not be readily accessible or cheap. Many researches have been conducted on the application of PGPR in agriculture crops and have found positive response of *Azotobacter* as biofertilizers to the plants. From this finding it can be suggested that *A.chroococcum* could be good biofertilizer as an alternative to nitrogen fertilizer.

6.2 RECOMMENDATIONS

1. Further research can be carried out to identify in a molecular level for a suitable and novel PGPR strain for bioinoculant production.
2. In pot experiment, this study is performed in normal conditions in outer environment. It should be done in green house for the better effective result of these bacteria towards the plant.
3. In this study only few growth parameters were observed, due to some circumstances full yield of tomato plants has not been observed. So, to insure the strong effectiveness of these bacteria, tomato plants can be observed until harvesting.
4. From this study, it is seen that *Azotobacter chroococcum* has positive effect in plants. So, to increase the use of biofertilizers for sustainable crop production technical training on the production approaches and advice as how the quality of PGPR production is carried out should be given to vegetable growers.

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APPENDIXES

APPENDIXCES

APPENDIX-A

Materials and equipment

List of the materials

1. Glass wares

Test tubes
Petri plates
Beaker
Eppendorf tube
Conical flasks
Glass slides
Micro pipette
Glass rod
Micropipette tips
Measuring cylinders

2. Miscellaneous

Inoculating loop
Gloves
Labeling sticker
Match box
Forceps
Test tube rack
Bunsen burner
Marker
Cotton swabs

3. Equipment

Autoclave
Hot air oven
Incubator
Microscope
Water bath shaker
Digital balance

4. Reagents/stains

Safranin
Alcohol
Gram's iodine
Lysol
Crystal violet
Hydrogen peroxide

5. Culture media

Agar powder
Beef extract
Nutrient broth
Starch agar
Peptone
Nutrient agar
Typtone

Biochemical media

Glucose
Urease
Mannitol
Maltose

APPENDIX-B

Culture media used in Research

1. Azotobacter Agar (Mannitol)

Ingredients	Gms/Litre
Dipotassium phosphate	1.000
Magnesium sulphate	0.200
Sodium chloride	0.200
Ferrous sulphate	TRACE
Soil extract	5.000
Mannitol	20.000
Agar	15.000
Final pH (at 25°C)	8.3±0.2

Suspend 41.4 grams in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes

2. Nutrient agar

Ingredient	Gms/Litre
Beef extract	0.5g
Yeast extract	1g
Peptone	2.5g
Distilled water	500mL

3. Peptone broth

Ingredients	Gms/Litre
Peptone	10.0
Sodium chloride	5.0
Final pH (at 25°C)	7.2±0.2

4. Starch Agar

Ingredients	**Gms/Litre**
Beef extract	3
Soluble starch	10
Agar	12
pH	7.3±0.2

Suspend 25gm of powder in 1 L of purified water and mix thoroughly. Heat and boil for 1 min, autoclave at 121°C for 15 min.

APPENDIX-C

Scientific Classification of *Azotobacter chroococcum* and phytopathogens used as model organisms.

Kingdom	**Bacteria**
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	Azotobaceriaceae
Genus	*Azotobacter*
Species	*chrococcum*

APPENDIX-D

Table 1: Morphological characteristics of isolates

S. N	Sample code	Gram stain	Cell shape	Colony shape margin	Color	Consistency	Motility	Catalase
1	S10A9	-ve	oval rod	Medium circular flat	Transparent	Dry	+	+
2	S5J3	-ve	oval rod	Medium circular flat	Transparent	Slimy	+	+
3	S2A1	-ve	oval rod in chain	Small circular raised	Brown	Viscous	+	+
4	S2A2	-ve	oval rod	Small circular raised	Brown	Viscous	+	+
5	S10A6	-ve	small oval rod in clustered	Small circular raised	Milky white	Mucoid	+	+
6	S3A4	-ve	oval rod	Medium circular flat	Creamy white	Dry	+	+
7	S20A1	-ve	oval rod in chain	Medium circular flat	Milky white	Mucoid	+	+
8	S20A5	-ve	oval rod in clustered	Small circular raised	Brown	Viscous	+	+
9	S18J3	-ve	oval rod in chain	Irregular flat	Creamy white	Mucoid	+	+
10	S25Aa	-ve	oval rod in clustered	Medium circular flat	Brown	Viscous	+	+
11	S12Ab	-ve	oval rod in chain	Medium circular flat	Brown	Viscous	+	+
12	S9Ac	-ve	oval rod mostly in pair	Medium circular flat	Brown	Viscous	+	+
13	S8Ja	-ve	small oval rod in clustered	Medium circular flat	Brown	Viscous	+	+
14	S7Jc	-ve	oval rod	Medium circular flat	Brown	Viscous	-	+
15	S20A6	-ve	oval rod in chain	Irregular flat	Brown	Mucoid	+	+
16	S20A3	-ve	oval rod	Irregular raised	Brown	Mucoid	+	+
17	S25a	-ve	small rod	large circular convex	Water droplet	Mucoid	+	+
18	S24b	-ve	small rod	large circular convex	Water droplet	Mucoid	+	+
19	S22a	-ve	small rod	large circular convex	Water droplet	Mucoid	+	+
20	S19Jd	-ve	oval rod in	Small	Brown	Dry	+	+

			chain	circular flat				
21	S4Jd	-ve	oval rod in clustered	Medium circular flat	Milky white	Mucoid	+	+
22	S21Ad	-ve	oval rod in chain	Medium circular flat	Yellow	Mucoid	+	+
23	S12Ae	-ve	oval rod in single and pair	Small circular flat	Pale brown	Dry	-	+
24	S8Je	-ve	oval rod in clustered	Medium circular flat	Yellow	Mucoid	+	+
25	S8Jf	-ve	oval rod in chain	Medium circular flat	Brown	Dry	+	+
26	S14Ag	-ve	small oval rod in clustered	Irregular raised	Creamy white	Mucoid	+	+
27	S23a	-ve	oval rod in chain	Small circular raised	Milky white	Mucoid	+	+
28	S12	-ve	oval rod shape	Medium circular flat	Milky white	Mucoid	+	+
29	S15a	-ve	oval rod in clustered	Medium circular flat	Milky white	Mucoid	-	+
30	S15b	-ve	oval rod in single and pair	Medium circular flat	Milky white	Mucoid	+	+
31	S1d	-ve	oval rod in single and pair	Medium circular flat	Milky white	Mucoid	+	+
32	S5Jj	-ve	oval rod in chain	Medium circular flat	Brown	Viscous	+	+
33	S5Jh	-ve	oval rod in clustered	Medium circular flat	Yellow	Mucoid	+	+
34	S235	-ve	oval rod in chain	Small circular raised	Transparent	Mucoid	+	+
35	S1a	-ve	oval rod in clustered	Medium circular flat	Transparent	Mucoid	+	+
36	S21Ae	-ve	oval rod in clustered	Medium circular flat	Pale brown	Mucoid	+	+

Table 2: Biochemical characteristics of isolates

S.N	Sample code	Motility	Mannitol	Maltose	Glucose	Starch hydrolysis
1	S10A9	+	+	+	-	+
2	S5J3	+	+	+	+	+
3	S2A1	+	+	+	+	+
4	S2A2	+	+	+	-	+
5	S10A6	+	+	+	+	+
6	S3A4	+	+	+	+	+
7	S20A1	+	+	+	-	+
8	S20A5	+	+	+	-	+
9	S18J3	-	+	+	+	+
10	S25Aa	+	+	+	+	+
11	S12Ab	-	+	+	-	+
12	S9Ac	+	+	+	+	+
13	S8Ja	+	+	+	+	-
14	S7Jc	+	-	+	+	+
15	S20A6	+	+	+	+	+
16	S20A3	-	+	+	-	+
17	S25a	+	+	+	+	+
18	S24b	+	+	+	+	-
19	S22a	+	+	+	+	-
20	S19Jd	+	+	+	+	+
21	S24Jd	+	+	+	+	+
22	S21Ad	+	-	+	-	+
23	S12Ae	+	+	+	+	+
24	S8je	+	-	-	+	+
25	S8Jf	+	+	+	+	+
26	S14Ag	+	-	+	+	+
27	S23a	+	-	-	-	+
28	S12	+	-	+	-	+
29	S15a	+	+	+	+	+
30	S15b	+	+	+	+	+
31	S1d	+	-	+	+	+
32	S5Jj	+	+	+	+	+
33	S5Jh	+	-	-	+	+
34	S235	+	+	+	-	+
35	S1a	+	-	-	-	+
36	S21Ae	+	+	+	+	+

Table 3: Physical and chemical properties of field soil use in pot experiment.

Physical properties of soil		Chemical properties of soil	
Moisture content	82%	Nitrogen	0.196%
Water holding capacity	23.97%	Potassium	96.4ppm
pH	7.8	Carbon	2.4%

APPENDIX-E

Statistical analysis of measured parameters of plants

Leaves- Treatments according to DAT

1. S-NI by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	11302.889	5651.44	110.0931	<.0001*
Error	6	308.000	51.33		
C. Total	8	11610.889			

Connecting Letters Report

Level			Mean
60	A		108.33333
40		B	42.66667
20		B	26.33333

Levels not connected by same letter are significantly different.

2. NS-NI by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	6814.8889	3407.44	12.1598	0.0077*
Error	6	1681.3333	280.22		
C. Total	8	8496.2222			

Connecting Letters Report

Level			Mean
60	A		128.00000
20	A		108.33333
40		B	62.33333

Levels not connected by same letter are significantly different.

3. S-I by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	16224.889	8112.44	29.5356	0.0008*
Error	6	1648.000	274.67		
C. Total	8	17872.889			

Connecting Letters Report

Level				Mean
60	A			135.33333
40		B		82.66667
20			C	31.33333

Levels not connected by same letter are significantly different.

4. NS-I by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	18284.667	9142.33	10.6900	0.0105*
Error	6	5131.333	855.22		
C. Total	8	23416.000			

Connecting Letters Report

Level			Mean
60	A		149.00000
40	A	B	90.33333
20		B	38.66667

Levels not connected by same letter are significantly different.

Leaves- DAT according to treatments

1. 20 DAT by treatments:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	13303.000	4434.33	66.1019	<.0001*
Error	8	536.667	67.08		
C. Total	11	13839.667			

Connecting Letters Report

Level			Mean
NS-NI	A		108.33333
NS-I		B	38.66667
S-I		B	31.33333
S-NI		B	26.33333

Levels not connected by same letter are significantly different.

2. 40 DAT by treatment:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	4136.3333	1378.78	6.6023	0.0148*
Error	8	1670.6667	208.83		
C. Total	11	5807.0000			

Connecting Letters Report

Level			Mean
NS-I	A		90.333333
S-I	A		82.666667
NS-NI	A	B	62.333333
S-NI		B	42.666667

Levels not connected by same letter are significantly different.

3. 60 DAT by treatment:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	2588.3333	862.778	1.0520	0.4212
Error	8	6561.3333	820.167		
C. Total	11	9149.6667			

Connecting Letters Report

Level		Mean
NS-I	A	149.00000
S-I	A	135.33333
NS-NI	A	128.00000
S-NI	A	108.33333

Levels not connected by same letter are significantly different.

RL & NC by treatment:

1. RL by treatments:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	27.870000	9.29000	9.9005	0.0045*
Error	8	7.506667	0.93833		
C. Total	11	35.376667			

Connecting Letters Report

Level			Mean
NS-I	A		14.633333
S-I	A	B	12.933333
S-NI		B	11.033333
NS-NI		B	10.933333

Levels not connected by same letter are significantly different.

2. NC by treatment:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Treatment	3	0.40666667	0.135556	3.6970
Error	8	0.29333333	0.036667	
C. Total	11	0.70000000		

Connecting Letters Report

Level		Mean
NS-I	A	1.9666667
NS-NI	A	1.9333333
S-I	A	1.8000000
S-NI	A	1.5000000

Levels not connected by same letter are significantly different.

Shoot length: Treatment by DAT

1. S-NI by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	88.66667	44.3333	1.1022	0.3911
Error	6	241.33333	40.2222		
C. Total	8	330.00000			

Connecting Letters Report

Level		Mean
60	A	29.000000
40	A	27.333333
20	A	21.666667

Levels not connected by same letter are significantly different.

2. NS-NI by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	380.6667	190.333	1.3137	0.3364
Error	6	869.3333	144.889		
C. Total	8	1250.0000			

Connecting Letters Report

Level		Mean
60	A	40.000000
40	A	34.666667
20	A	24.333333

Levels not connected by same letter are significantly different.

3. S-I by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	248.22222	124.111	2.8136	0.1374
Error	6	264.66667	44.111		
C. Total	8	512.88889			

Connecting Letters Report

Level		Mean
60	A	41.000000
40	A	38.000000
20	A	28.666667

Levels not connected by same letter are significantly different.

4. NS-I by DAT:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
DAT	2	757.5556	378.778	1.4796	0.3004
Error	6	1536.0000	256.000		
C. Total	8	2293.5556			

Connecting Letters Report

Level		Mean
60	A	54.666667
40	A	45.666667
20	A	32.333333

Levels not connected by same letter are significantly different

Shoot length- DAT according to treatments

1. 20 DAT by treatments:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	199.5833	66.528	0.6043	0.6304
Error	8	880.6667	110.083		
C. Total	11	1080.2500			

Connecting Letters Report

Level		Mean
NS-I	A	32.333333
S-I	A	28.666667
NS-NI	A	24.333333
S-NI	A	21.666667

Levels not connected by same letter are significantly different.

2. 40 DAT by treatment:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	520.9167	173.639	1.1832	0.3755
Error	8	1174.0000	146.750		
C. Total	11	1694.9167			

Connecting Letters Report

Level		Mean
NS-I	A	45.666667
S-I	A	38.000000
NS-NI	A	34.666667
S-NI	A	27.333333

Levels not connected by same letter are significantly different.

3. 60 DAT by treatment:

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	995.0000	331.667	3.0973	0.0893
Error	8	856.6667	107.083		
C. Total	11	1851.6667			

Connecting Letters Report

Level		Mean
NS-I	A	54.666667
S-I	A	41.000000
NS-NI	A	40.000000
S-NI	A	29.000000

Levels not connected by same letter are significantly different.