GROWTH PROMOTIONAL EFFECT OF *Azotobacter chroococcum* ON *Abelmoschus esculentus* (OKRA) AND ITS ANTAGONISTIC ACTIVITIES AGAINST SOME SELECTED PHYTOPATHOGENS

A

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 **Mrs. Uma Khatri** has completed this dissertation work entitled **"Growth promotional effect of *Azotobacter chroococcum* on *Abelmoschus esculentus* (Okra) and its antagonistic activities against some selected phytopathogens"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (**Agriculture**) under my supervision. To our knowledge, this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Assistant Prof. Mr. Hemanta Khanal** this dissertation work of **Mrs. Uma Khatri** entitled **"Growth promotional effect of *Azotobacter chroococcum* on *Abelmoschus esculentus* (Okra) and its antagonistic activities against some selected phytopathogens"** 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

Azotobacter chroococcum is an aerobic, free-living, ubiquitous, heterotrophic, non- symbiotic biological nitrogen fixer present in soils. *A. chroococcum* is plant growth promoting rhizobacteria (PGPR) that aggressively colonize the root zone and promote plant growth, protect the plants from phytopathogens and also act as biocontrol agents. *A. chroococcum* synthesizes and secretes considerable amount of biologically active substances such as vitamins (riboflavin), amino acids (thiamine), plant growth hormones (nicotine, indole acetic acid, and gibberellins), antifungal compounds (HCN), and siderophores. The aim of study is to isolate and identify *A. chroococcum* from rhizospheric soil of Eastern Nepal to observe its effect on growth of okra plant and its antagonistic activity against some phytopathogens. The rhizospheric soil was collected from five places of Eastern Nepal, in sterile plastic bag and cultured in mannitol N-free agar media by serial dilution and incubated at 25°C for 48 hrs. Morphological, cultural, biochemical and physiological characterization was done and bacteria were identified. Isolated *A. chroococcum* were cultured in mannitol N-free broth and inoculated on okra plants by seed inoculation technique. It was found that okra plant inoculated with *A. chroococcum* was improved in growth parameter in height, root length, fresh and dry compared to inoculated plant. Increasement in height of plants was found to be around 21.33% comparing to that of uninoculated plants. No significant difference was found on number of leaves in between treatments and control plants. In vitro effects on selected phytopathogens were observed on modified mannitol N-free agar by dual culture method. Similarly, different isolates were found to suppress plant pathogens being the AD most potent in suppressing *R. solani* and *F. oxysporum* by 38.5% and 24.9% and AB being most potent in suppressing *S. rolfsii* by 35.5%. while other isolates were found to be moderate potent.

Key words: *A. chroococcum*, PGPR, HCN, Phytopathogens, siderophores

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BCA	-	Biological Control Agent
IP	-	Inhibition percentage
CRD	-	Completely Randomized block Design
DAI	-	Days After Inoculation
DAT	-	Days After Transplantation
DI	-	Disease Incidence
HCN	-	Hydrogen cyanide
FO	-	*Fusarium oxysporum*
IAA	-	Indole Acetic Acid
PDA	-	Potato Dextrose Agar
ISR	-	Induced systemic resistance
CMC	-	Carboxy Methyl Cellulose
PGPR	-	Plant Growth Promoting Rhizobacteria
NA	-	Nutrient Agar
RS	-	*Rhizoctonia solani*
LPS	-	Lipopolysaccharides
SR	-	*Sclerotium rolfsii*
PSB	-	Phosphate-solubilizing bacteria
BTB	-	Bromothymol blue

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CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

The utilization of plant growth promoting rhizobacteria (PGPR) in agriculture is continuously increasing as it offers an effective tool to replace the use of chemical fertilizers, pesticides and other harmful supplements (Ansari et al 2017; Ansari and Mahmood, 2019ab).

Growth promoting substances are produced in huge quantities by the action of these rhizosphere microorganisms that directly or indirectly influence the overall morphology and physiology of the crops. Recent advances in the field of sustainable development relies on the use and diversity of PGPR, their colonizing capability and the mechanism of action that may be used to facilitate their application as a dependable element in the management of sustainable agricultural system (Bhattacharyya and Jha, 2012; Di Benedetto et al 2017; Ansari and Mahmood 2019a, b).

Plant pathogen is an organism that causes a disease on a plant. Plant pathogens are forming a huge problem on the economic and life stability. The plant pathogens cause the diseases for leaf, stem, root, vascular system and fruit. Application of these chemical pesticides is often failing and even raises several problems either in human life, environment or agricultural products (Gamliel et al 1997).

In the present context, the best alternative of chemical pesticides or non-chemical means of plant disease control that can be used to protect crops from soil-borne pathogens is introduction of biocontrol agents (Ahmad et al 2005).

A. chroococcum is regarded as free-living, ubiquitous, aerobic nitrogen fixer present in soils. They are oval or spherical in shape and form thick-walled cysts (dormant cells resistant to deleterious conditions) under unfavorable environmental conditions. Being the major group of soil borne bacteria have significant influence on soil physiological and structural properties. Also help

to replace chemical fertilizer for the sustainable agriculture production by fixing the atmospheric nitrogen and producing growth-promoting substances (Lenart 2012).

Besides nitrogen fixation, *Azotobacter* synthesizes and secretes considerable amount of biologically active substances like B-vitamins, nicotinic acids, pantothenic acids, biotin, heteroauxins, gibberellins, etc., which are reported to enhance the growth of plants and their tolerance to pathogenic diseases (Van Loon 2007). Research on *A. chroococcum* in crop production has shown its importance in improving plant nutrition and amelioration of soil fertility (Kurrey et al 2018). Several strains of Azotobacter are found to be able to produce amino acids when grown in culture media supplemented with various carbon and nitrogen sources (González-López et al 2005). Such substances produced by these rhizobacteria are implicated in several processes thus leading to plant-grown promotion (Jnawali et al 2015). The scope of utilizing *A. chroococcum* in research experiments as microbial inoculant through release of growth substances and their impact on the plant has markedly improved crop production in agriculture (Gothandapani et al 2017).

Various crops in India have been inoculated with diazotrophs, especially *Azotobacter* spp., which excretes ammonia in the rhizosphere in presence of root exudates and helps in modification of nutrient uptake by the plants (Narula et al 1981). *Azotobacter* is known to produce secondary metabolites such as vitamins (riboflavin), amino acids (thiamine), plant growth hormones (nicotine, indole acetic acid, and gibberellins), antifungal compounds, and siderophores, and importantly they can fix atmospheric-free nitrogen (Myresiotis et al 2012). These growth- promoting substances have direct influence on shoot and root length as well as seed germination of several agricultural crops (Ahmad et al 2005). These secondary metabolites influence plant growth promotion by excreting vitamins, amino acids, and auxins. Siderophores can provide iron to plants and polyhydroxy butyrate (PHB) which can be used in large-scale production of alginic acid. Antifungal compounds, HCN, can inhibit the pathogenic organisms in plant rhizosphere.

Azotobacter species improves seed germination and plant growth (Singh 2002).

They pose advantageous impacts on the crop growth and yield through the biosynthesis of biologically active substances, instigation of rhizospheric microbes, production of phytopathogenic inhibitors, alteration of nutrient uptake and eventually magnifying the biological nitrogen fixation (Lenart 2012).

Azotobacter species act as biocontrol agents for many plant pathogens. *A. chroococcum* inhibit the growth of *Aspergillus, Alternaria, F. oxysporum,* and *R. solani* and they are known to produce antimicrobial agents such as 2,3-dihydroxybenzoic acid, aminochelin, azotochelin, protochelin, and azotobactin (Kraepiel et al 2009). The species of *Azotobacter* and *Arthrobacter* inhibit root colonization of *F. verticillioides* and suppress fumonisin B-1 production by *A. armeniacus*. Antifungal activity of *A. vinelandii* against *F. oxysporum* showed maximum zone of inhibition (40 mm) which was known to cause diseases of agricultural crops, viz., chili and pigeon pea (Chennappa et al 2014a; Cavaglieri et al 2005; Bhosale et al 2013). *Azotobacter* can provide protection against drought and produces antifungal antibiotic substance which inhibits the growth of soil borne fungi such as *Aspergillus, Fusarium, Curvularia, Alternaria,* and *Helminthosporium* (Khan et al 2008; Mali and Bodhankar, 2009; Agarwal and Singh, 2002).

Okra (*Abelmoschus esculentus L. Moench)* is an important summer vegetable of Nepal which also known as Ladies Fingers is a member of Malvaceae family (Maurya et al,2013). Locally it is known as ‘Bhindi’ or ‘Chiple bhindi’. It is an important vegetable crop in the tropical and sub-tropical regions in the world (Osekita 2009). It probably originated in either tropical Africa or tropical Asia, and is now widely grown throughout the tropics (Ali et al 2012). The crop is well distributed throughout the Indian subcontinent and East Asia (Rashid 1999). Okra is a multipurpose crop. Okra is the rich source of carbohydrate, amino acids, vitamin which have multipurpose use like fresh or cooked consumption, as fodder to animal, medicinal and industrial use (Farinde et al., 2007; Kumar et al., 2017) The tender okra pods are consumed fresh as well as canned and dried.

Okra seeds are roasted, grounded and used as substitute of coffee in Turkey. It is a nutritious and delicious vegetable, fairly rich in vitamins and minerals (Khushk, Usman, & Memon, 2003). The edible portion of pod (100g) has moderate levels of vitamin A (375 IU), vitamin C (21.1mg), calcium (81mg), phosphorus (63mg) and potassium (303mg). (USDA National Nutrient Database) The content of thiamine (0.07mg), riboflavin (0.08mg) and niacin (0.08mg) per 100gm edible portion of pod is higher than that of many vegetables (Rashid 1999).

It is a widely cultivated food crop and is globally known for its palatability. The immature green pods of okra are usually consumed as vegetables, while the extract of the pods also serves as a thickening agent in numerous recipes for soups, as well as sauces, to augment their viscosity (Dhaliwal M.S., 2010, Kumar A et al ,2013). Another noteworthy application of okra fruit is their wide use in the pickle industry. The polysaccharides present in okra are used in sweetened frozen foods such as ice-creams, as well as bakery products, due to their health benefits and longer shelf-lives (Archana G et al ,2015, Costantino A et al,2004, Yuennan P et al ,2014).

Okra is a popular vegetable crop with good nutritional significance, along with certain therapeutic values, which makes it a potential candidate in the use of a variety of nutraceuticals. Different parts of the okra fruit (mucilage, seed, and pods) contain certain important bioactive components, which confer its medicinal properties. The phytochemicals of okra have been studied for their potential therapeutic activities on various chronic diseases, such as type-2 diabetes, cardiovascular, and digestive diseases, as well as the antifatigue effect, liver detoxification, antibacterial, and chemo-preventive activities. Moreover, okra mucilage has been widely used in medicinal applications such as a plasma replacement or blood volume expanders (Balakumar P et al,2012, Lv M et al 2015, Ge J et al,2016).

1.2 Objectives

1.2.1 General objectives

To study growth promotional effect of *A. chroococcum* on plant(okra) and its antagonistic activity against some selected Phyto-pathogens.

1.2.2 Specific objectives

1. To identify *A. chroococcum* from soil of different geographical soil samples.
2. To analyze Hydrogen cyanide production (HCN) and indole acetic acid (IAA) production by *A. chroococcum.*
3. To evaluate various agronomic parameters of *A. chroococcum* inoculated and non-inoculated plant.
4. To determine the antagonistic effect of against some selected Phyto-pathogens.
5. To determine ammonia production by *A. chroococcum*.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction of *Azotobacter chroococcum*

The genus *Azotobacter*, belonging to the family Azotobacteriaceae is an aerobic, heterotrophic, non- symbiotic biological nitrogen fixing microbe. It has been found that some *Azotobacter* species exist in association with some crops especially cereals. There are around six species in the genus *Azotobacter* some of which are motile by means of peritrichous flagella; others are not (Martyniuk et al 2003). *A. chroococcum* are most commonly inhabiting many soils all over the world (Mahato et al 2009). The *Azotobacter* genus was discovered in 1901 by Dutch microbiologist and botanist Martinus Beijerinck. *A. chroococcum*is the first aerobic, free-living nitrogen fixer discovered (Beijerinck et al 1901).

Azotobacter represents the main group of heterotrophic free-living nitrogen-fixing bacteria. They are ubiquitous and abundantly found in neutral to weakly acidic soils. In dry soils, *Azotobacter* can survive in the form of cysts for up to 24 years (Moreno et al 1986). *Azotobacter* sp. is generally present in natural and alkaline soil with its most commonly occurring species found in arable soils. They are typically polymorphic and their size ranges from 2-10µm long and 1-2µm wide (Salhia 2013). The free-living, gram-negative, motile and mesophilic *Azotobacter* spp. are capable of fixing on an average 20kg N/ha/per year (Rawia et al 2009). These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis. This cell protein is then mineralized in soil after the death of *Azotobacter* cells thereby contributing towards the nitrogen availability of the crop plants. *Azotobacter spp*. is sensitive to acidic pH, high salts, and temperature (Tchan et al 1989). *Azotobacter has* beneficial effects on crop growth and yield through, biosynthesis of biologically active substances, stimulation of rhizospheric microbes, producing phytopathogenic inhibitors (Lenart 2012). *Azotobacter*, widely used as biofertilizer, binds atmospheric nitrogen and release it in the form of ammonium ions into the soils (Salhia 2013).

2.2 Taxonomic classification

The taxonomic classification of *A. chroococcum* and soil borne phytopathogens used as model organism in this study are listed in Appendix III.

2.3 Morphological characters

A. chroococcum is a Gram negative, microaerophilic, bacillus in shape and is that belongs to the *Azotobacter* genus. It is mesophilic, grows best in moderate-temperature soils 25-30°C and requires a neutral pH environment, able to fix nitrogen under aerobic conditions. The soil cannot be poor in phosphorus or else nitrogen fixing can be hindered. And in addition to phosphorus these bacteria needed potassium, Sulphur, magnesium, and calcium to grow. It also forms the dark-brown, water-soluble pigment melanin at high levels of metabolism during the fixation of nitrogen, which is thought to protect the nitrogenase system from oxygen. In the *Azotobacter* selective medium, these bacteria appear as cream-colored colonies, Gram negative bacilli, large and short, in pairs or in chains, irregular and bright colonies. The colonies appear slightly viscous, semi-transparent during the early growth and later changes to dark brown and also form cysts.

A cyst of the genus *Azotobacter* is the resting form of a vegetative cell. *Azotobacter* does not form endospores but form thick-walled cysts (means of asexual reproduction under favorable condition) (Salhia 2013). The formation of cysts is induced by changes in the concentration of nutrients in the medium and addition of some organic substances such as ethanol, n-butanol, or β-hydroxybutyrate. The formation of cysts is also induced by chemical factors and is accompanied by metabolic shifts, changes in catabolism, respiration and bio syn-thesis of macromolecules (Sadoff 1975).

2.4 *Azotobacter* as Plant growth promoting rhizobacteria (PGPR)

A. chroococcum is mainly identified as plant growth promoting rhizobacteria (PGPR) have more influence on the growth and yield of the crops. The

positive effects of PGPR are normally divided into two categories: growth promotion and biological control (Kleopper 1997). PGPR can help to replace nitrogen from chemical fertilizer for the sustainable cultivation by fixing the atmospheric N_2 and producing PGPS (Ahmad et al 2005). *A. chroococcum* is known to produce secondary metabolites such as vitamins (riboflavin), amino acids (thiamine), plant growth hormones (nicotine, indole acetic acid, and gibberellins), antifungal compounds, and siderophores, and importantly they can fix atmospheric-free nitrogen (Myresiotis et al 2012). Phytohormones (auxin, cytokinin, gibberellin) can stimulate root development. These growth-promoting substances have direct influence on shoot and root length as well as seed germination of several agricultural crops (Ahmad et al 2005). These hormonal substances, which originate from the rhizosphere or root surface, affect the growth of the closely associated higher plants (Ahmad et al 2005).

Siderophores can provide iron to plants and polyhydroxy butyrate (PHB) which can be used in large-scale production of alginic acid. Antifungal compounds, HCN, can inhibit the pathogenic organisms in plant rhizosphere. *Azotobacter* species improves seed germination and plant growth.

In past years, *Azotobacter sp*. had drawn a worldwide attention because of production of secondary metabolites such as siderophore, antibiotics, enzymes and phytohormones and involving in nitrogen fixation. Large number of field trials and various experiments carried throughout India and whole world have convincingly established the importance of *Azotobacter* as microbial inoculant. Various crops like wheat, barley, maize, sugar beet, carrot, cabbage, potato was inoculated with *Azotobacter* in during its. Brakel and Hilger, (1965) have showed that *Azotobacter* produced indol-3-acetic acid (IAA) when tryptophan was added to the medium. Hennequin and Blachere, (1966) have found only small amounts of IAA in old cultures of *Azotobacter* to which no tryptophan was added.

Azotobacter synthesize auxins, cytokinin, and GA-like substances, and these growth materials are the primary substance controlling the enhanced growth of tomato (Azcorn and Barea, 1975). Puertas and Gonzales, (1999) have report that dry weight of tomato plants inoculated with *A. chroococcum* and grown in

phosphate-deficient soil was significantly greater than that of non-inoculated plants.

Eklund (1970) has demonstrated that the presence of *A. chroococcum* in the rhizosphere of tomato and cucumber is correlated with increased germination and growth of seedlings. A study conducted by (Govedarica et al 1993) on the production of growth substances by nine *A. chroococcum* strains isolated from a chernozem soil has showed that these strains have the ability to produce auxins, gibberellins, and phenols and in association with the tomato plant, increase plant length, mass and nitrogen content. Vijayan et al (2007) have observed that foliar application of *A. chroococcum* to mulberry grown under saline soil conditions showed significant level of improvement in biochemical and morphological parameters of leaf. Under greenhouse conditions inoculation of *A. chroococcum* recorded a significant N and P uptake in both seed and stover in brown sarson (*Brasssica campestris*) over the control also increase in plant height, leaf number/plant, number of primary and secondary branches/plant, fresh and dry weight of whole plant (Wani 2012).

2.5 A. chroococcum as Biocontrol Agents

Azotobacter strain is one of the most active bio-control agents. Bio-control by using antagonistic microorganisms is a potential alternative to chemical compounds for crop protection against phytopathogens (ElKatatany et al 2003). The mechanisms through which *Azotobacter spp.* control plant diseases involve competition for niches and nutrients antibiosis, predation, and induction of plant defense responses. the production of siderophores, antimicrobial substances, toxins and also the growth hormones like auxins, gibberellins and cytokinin depending upon the bacterial strain, environmental conditions, pathogen involved and also the target. Such strategies used by the bacteria have been demonstrated to impart major resistance towards the attack of the plant pathogens. *Azotobacter* spp. can also produce antifungal compounds to fight against many plant pathogens (Jen-Hshuan 2006).

It has been observed that the antagonistic activity also conferred through the action of siderophores produced from *Azotobacter* spp. called Azotobactin.

Azotobactin shows antibacterial effect against root colonizing bacterial and fungal pathogens (Schalk 2008). These had been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria (Gupta et al 2001). *Azotobacter* is reported to produce an antibiotic having similar structure as that of azinomycin, which is well established fungicidal antibiotic. *Azotobacter* sp. by itself has utility as an extremely economical and eco-friendly bio pesticide (Rachin and Ahmed, 2005).

Some examples of the pathogens that have been managed by the use of *Azotobacter* as a bioinoculant includes *Alternaria*, *Fusarium, Rhizoctonia, Macrophomina, Curvularia, Helminthosporium* and *Aspergillus* (Jnawali et al 2015). *A. chroococcum* produces an antibiotic which inhibits the growth of several pathogenic fungi in rhizosphere thereby seedling mortality (Subba Rao 2001). Maheshwari et al (2012) have demonstrated that the strain TRA2 of *A. chroococcum* which is an isolate of wheat rhizosphere showed strong antagonistic activity against root rot fungus *Macrophomina phaseolina* and *Fusarium oxysporum*, also provided good protection to the plants by aggressively colonizing the roots of wheat crops. Akram et al (2016) have found that disease incidence by root-knot nematode *Meloidogyne incognita* was significantly reduced when *A. chroococcum* was applied to chickpea plants. Incidence of some diseases of mustard and rapeseeds could be reduced by inoculating with *Azotobacter* (Singh and Dutta, 2006).

Dual inoculation of *Azotobacter* and *Azospirillum* showed synergistic effects by improving growth prompting hormones, controlling pathogenesis and growth reducing agents due to producing fungicide antibiotics and compounds (antagonistic effect) and also air molecular N fixing and also producing growth prompting hormones such as auxin, cytokinin and gibberellins and solving mineral compound (Naseri et al 2013).

2.6 Mode of action

A. chroococcum as PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory

effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick 2012).

2.6.1 Direct mechanism

2.6.1.2 Nitrogen fixation

Nitrogen fixation comes among the most important biological processes and is considered as an interesting microbial activity on the earth's surface as it provides a way of recycling the nitrogen and plays an important role in nitrogen homeostasis in the biosphere (Wani et al 2016). Biological nitrogen fixation also helps in maintaining soil fertility and improving crop productivity (Vance and Graham, 1995).

Azotobacter spp. are non-symbiotic heterotrophic bacteria and capable of fixing about 20kg N/ha/per year (Kizilkaya 2009) and it can be used in crop production as a substitute for a portion of mineral nitrogen fertilizers (Hajnal et al 2004). *Azotobacter* is able to convert atmospheric nitrogen to ammonia, which in turn is taken up and utilized by the plants (Prajapati et al 2008). Such bacteria are immensely resistant to oxygen during nitrogen fixation due to respiration protection of nitrogenase (Hakeem et al 2016). In addition to the respiratory protection there also exist hydrogenase uptake as well as switch on–off mechanisms for the protection of nitrogenase enzyme from oxygen (Chhonkar et al 2009). Uptake of hydrogenase is involved in the metabolism of hydrogen (H_2) released during the process of nitrogen fixation (Partridge and Yates, 1982).

Romero-Perdomo et al (2017) have reported that the application of mixed culture of *Azotobacter* strains could reduce the need of N-fertilizers up-to 50%.

2.6.1.2 Phosphate solubilization

Phosphorus (P) is one of the major plant nutrients limiting plant growth. Soil microorganisms play a significant role in mobilizing P for plants by bringing about changes in pH in rhizospheric soil and also by producing chelating substances, which lead to solubilization of phosphates (Halder et al 1991). The

release of insoluble and fixed forms of P carried out by the action of phosphate-solubilizing bacteria (PSB) via the secretion of low molecular weight organic acids mainly gluconic and keto-gluconic acids and phosphatases (Rodriguez et al 1999; Chung et al 2005; Sashidhar et al 2010).

Kpomblekon and Tabatabai, (1994) have found that aliphatic acids with beta-hydroxyl and alpha-carboxyl groups, such as citric and oxalic acid, were very effective in solubilizing rock phosphate. Studies of phosphate solubilization by low-molecular weight organic acids were reviewed by Earl et al (1979) and Illmer et al (1995) and a large number of phosphate-solubilizing bacteria (PSB) have been isolated from the rhizosphere of several crops. The capability of P-solubilizing rhizobacteria to produce plant-growth-regulating substances of the auxin type may contribute to their stimulating effects on plant growth (Sattar and Gaur, 1987; Leinhos 1994; Leinhos and Bergmann, 1995). The phytohormone indole-3 acetic acid affects root elongation and lateral-root formation (Pilet and Saugy, 1987).

2.6.1.3 Siderophore production

Siderophores constitute a group of iron (Fe) chelating molecules that alter the availability of Fe in the extracellular medium through its ability to outcompete other natural ligands (Wichard et al 2009). Microbes utilize siderophores to reach the important iron resources in the environment. Bacteria belonging to genus *Azotobacter* express iron-rich nitrogenases, through which they reduce nitrogen (Baars et al 2016). *Azotobacter* spp. gain access to the sparingly soluble Fe in the environment by making Fe-siderophore complex and then this complex is absorbed by membrane bound receptors (Palanché et al 2004). Such Fe-siderophore complexes may not be available to other competing microorganisms thereby they may show anti-phytopathogenic activities and can directly improve plant growth by protecting plants from the pathogens attack (Hayat et al 2010).

siderophores produced by *A. vinelandii* also consists the ability to bind metals other than Fe and allow the uptake of additional metals like molybdenum (Mo) or vanadium (V) that are needed in nitrogenases (Bellenger et al 2008) and

also to take up toxic heavy metals like W and Zn (Huyer and Page, 1988; Kraepiel et al 2009). Siderophores of *A. vinelandii* have also been reported to help to flourish some freshwater algae in co-culture (Villa et al 2014). *A. chroococcum* is also reported to produce vibrioferrin and amphibactins in addition to a novel family of siderophores, the croch``````````````elins.

2.6.1.4 Phytohormone production

*Azotobacter s*pp. have also been noted for their ability to produce different growth hormones (IAA and other auxins, such as gibberellins and cytokinin) (Barea and Brown, 1974), vitamins, antibacterial and antifungal compounds and siderophores (Pandey and Kumar, 1989b) which directly or indirectly effect the plant growth and microbial activity also have stimulatory or inhibitory effects on certain physiological and biochemical processes in plants and microorganisms. These hormonal substances which originate from the rhizosphere or root surface affect the growth of the closely associated higher plants (Pandey and Kumar, 1989b).

Brakel and Hilger, (1965) have showed that *Azotobacter* produced indol-3-acetic acid (IAA) when tryptophan was added to the medium while as Hennequin and Blachere, (1966) have found only small amounts of IAA in old cultures of *Azotobacter* to which no tryptophan was added. Substances like amino acid produced by these rhizobacteria are involved in many processes that explain plant-grown promotion. Biochemical analysis of chlorophyll, nitrogen, phosphorous, potassium and protein content were higher in *Azotobacter* inoculated plants as compared to no inoculated control plants (Naseri et al 2013).

2.6.2 Indirect mechanisms

The major indirect mechanism of plant growth promotion is through acting as biocontrol agents (Glick 2012). In general, competition for nutrients, niche exclusion, induced systemic resistance and antifungal metabolites production are the chief modes of biocontrol activity in PGPR (Lugtenberg and Kamilova, 2009).

Azotobacter spp have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, viscosinamide and tensin (Bhattacharyya and Jha, 2012). Interaction of some rhizobacteria with the plant roots can result in plant resistance against some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009). ISR involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plant's defense responses against a variety of plant pathogens (Glick 2012). Many individual bacterial components induce ISR, such as lipopolysaccharides (LPS), flagella, siderophores, cyclic lipopeptides, 2,4-diacetylphloroglucinol, homoserine lactones, and volatiles like, acetoin and 2,3-butanediol (Lugtenberg and Kamilova, 2009).

2.7 Soil Borne Phytopathogens used as Model Organisms

Azotobacter is also known to be associated with the suppression of pathogenic diseases of plants. Several examples are present in the literature advocating the importance of disease suppression by different species of *Azotobacter.*

Several mechanisms can be implicated behind the management strategies used by the bacteria for the control of plant diseases. These may include the production of siderophores, antimicrobial substances, toxins and also the growth hormones like auxins, gibberellins and cytokinin.

Seedlings of economically important crop plants are attacked by various soil-borne pathogenic fungi, such as *Pythium, Fusarium, Rhizoctonia, Phytophthora* and others, which cause either seed rot before germination or seedling rot after germination, resulting in billions of dollars in cumulative crop losses (Gohel et al 2006). These diseases are often termed pre- and post-emergence damping-off, or seedling blight. Greenhouse crops grown in soil-less cultures, as well as field crops, are susceptible to soil-borne fungal pathogens, resulting in considerable economic losses.

This dissertation focuses on the application of *A. chroococcum* in biological control of following phytopathogens.

2.7.1. *Rhizoctonia solani*

Rhizoctonia solani is one of the most important ubiquitous soil-borne fungal pathogens, causing diseases in different crops such as rice, bean and tomato. *R. solani* also attacks cotton seedlings causing pre- or post-emergence damping-off, which often results in substantial losses (Brown and McCarter, 1976).

Treatment of seeds with *Azotobacter* and *Azospirillum* can help to control disease incidence and severity, improve nutrient uptake efficiency, produce thiamin, riboflavin, indole acetic acid and gibberellins, and promote growth leading to enhanced yield (Bahrani et al 2010). Biari et al (2008) has found that treatment with plant growth-promoting rhizobacteria belonging to the genera *Azospirillum* and *Azotobacter* significantly increased plant height, shoot and seed dry weight, ear dry weight and length and number of seeds per row, in *Zea mays*, at research farm of Shahrood University of Technology, Iran (2006).

2.7.2 *Fusarium oxysporum*

The species *Fusarium oxysporum* is well represented among the communities of soil borne fungi, in every type of soil all over the world (Burgess 1981). *F. oxysporum* is a major soil borne pathogen and responsible for causing wilt diseases on a variety of crop plants (Nelson et al 1981). *F. oxysporum* distributed in soils throughout the worlds, and showing symptoms consisted of wilting of foliage (drying and withering of older leaves), stunting of plants, and reduced fruit production. At severe stage plants eventually collapsed and died. Internal vascular and cortical tissues of plant crowns showed a brown to orange brown discoloration. *F. oxysporum* is the most frequently occurring and damaging strawberry plants infected by crown and root diseases (Koike et al 2009; Fang et al 2011a; Fang et al 2011 b, Fang et al 2013; Koike et al 2013).

2.7.3 *Sclerotium rolfsii*

Sclerotium rolfsii is soil-borne saprophytic fungus which causes different types of diseases like collar-rot, *sclerotium* wilt, stem-rot, charcoal rot, seedling blight, damping-off, foot-rot, stem blight and root-rot in more than 500 plants species including tomato, chilly, sunflower, cucumber, brinjal, soybean, maize, groundnut, bean, watermelon etc.

Seedling blight, or damping off, is a disease complex caused by several seed-borne and soil-borne fungi, including species of *Cochiobolus, Curvularia, Fusarium, Rhizoctonia* and *Sclerotium.* Typically, the rice seedlings are weakened or killed by the fungi. Seedling blight causes stands of rice to be spotty, irregular and thin. Fungi enter the young seedlings and either kill or injure them. Blighted seedlings that emerge from the soil die soon after emergence. Those that survive generally lack vigor, are yellow or pale green and do not compete well with healthy seedlings

The soil-borne seedling blight fungus, *Sclerotium rolfsii,* kills or severely injures large numbers of rice seedlings after they emerge if the weather at emergence is humid and warm. A cottony white mold develops on the lower parts of affected plants. Seeds that carry blight fungi frequently have spotted or discolored hulls, but seed can be infected and still appear to be clean.

2.8 Okra plant

Okra (*Abelmoschus esculentus*) is an important summer vegetable of Nepal which belongs to family Malvaceae (Maurya et al 2013). Locally it is known as ‘Bhindi’ or ‘Chiple bhindi’. It was originated from Africa and spread to tropics, subtropics and warmer part of temperate region. Okra is a multipurpose crop. The tender okra pods are consumed fresh as well as canned and dried. Okra seeds are roasted, grounded and used as substitute of coffee in Turkey. It is a nutritious and delicious vegetable, fairly rich in vitamins and minerals (Khushk, Usman, & Memon, 2003). Okra is the rich source of carbohydrate, amino acids, vitamin which have multipurpose use like fresh or cooked consumption, as fodder to animal, medicinal and industrial use (Farinde et al 2007; Kumar et al 2017). The tender fruits are cooked as

vegetables. It contains vitamin A, B and C with little iron. Matured fruits and stem containing crude fiber are used in the paper industry. In some places, the plants are soaked in water and the resulting solution is used as clarifier in the manufacture of jiggery.

Okra is an annual shrub that is cultivated mostly within tropical and subtropical regions across the globe and represents a popular garden crop, as well as a farm crop. It is a widely cultivated food crop and is globally known for its palatability. The immature green pods of okra are usually consumed as vegetables, while the extract of the pods also serves as a thickening agent in numerous recipes for soups, as well as sauces, to augment their viscosity (Dhaliwal M.S., 2010, Kumar A et al ,2013). Another noteworthy application of okra fruit is their wide use in the pickle industry. The polysaccharides present in okra are used in sweetened frozen foods such as ice-creams, as well as bakery products, due to their health benefits and longer shelf-lives (Archana G et al ,2015, Costantino A et al,2004, Yuennan P et al ,2014).

Okra is a popular vegetable crop with good nutritional significance, along with certain therapeutic values, which makes it a potential candidate in the use of a variety of nutraceuticals. Different parts of the okra fruit (mucilage, seed, and pods) contain certain important bioactive components, which confer its medicinal properties. The phytochemicals of okra have been studied for their potential therapeutic activities on various chronic diseases, such as type-2 diabetes, cardiovascular, and digestive diseases, as well as the antifatigue effect, liver detoxification, antibacterial, and chemo-preventive activities. Moreover, okra mucilage has been widely used in medicinal applications such as a plasma replacement or blood volume expanders (Balakumar P et al,2012, Lv M et al 2015, Ge J et al,2016). Overall, okra is considered to be an easily available, low-cost vegetable crop with various nutritional values and potential health benefits.

CHAPTER III

MATERIALS AND METHODS

This study was carried out in microbiology laboratory of Central Campus of Technology, Dharan and at my garden Itahari. The laboratory techniques were according to the standard methods.

3.1 Materials Required

The materials, equipment, media and reagents used and their application in this study are systematically listed in Appendix I.

3.2 Methods

3.2.1 Study design

The study was conducted from July 2018 to July 2019. This study was a laboratory based cross-sectional study. All the work concerning this research was carried out in microbiology laboratory of Central Campus of Technology, Dharan and at my garden Itahari.

3.2.2 Soil sampling

In this study, soil samples (50g each) were collected randomly from different ecological habitat (agricultural area) of eastern Nepal for the isolation of *A. chroococcum*. The samples were collected from top 2-5cm depth of rhizospheric soil. The soil samples were collected from particular field in the polythene bag labeled respectively and transported within 1 hour as possible in lab. The samples were stored at 4°C in the laboratory.

3.2.3 Laboratory set up

Laboratory setting was done in microbiology laboratory, Central Campus of Technology, Dharan.

3.2.4 Cleaning and sterilization of glass wares

The Petri plates, pipettes, conical flasks, test tubes, beakers etc. used in the experiments were thoroughly washed and dried. The Petri-plates and pipettes

were wrapped in a silver foil and sterilized in hot air oven at 160ºC for 2 hours (Aneja 2004).

3.3 Isolation of *A. chroococcum*

10gm of soil sample was taken and added to 90ml of sterilized distilled water to make dilution of 10^{-1}. Five-fold serial dilution of each soil samples were prepared in sterilized distill water and 0.1ml of each dilution i.e., 10^{-4} and 10^{-6} dilutions were poured onto Mannitol N-free Agar contained in a Petri plate and spread uniformly with the help of L-shaped dolly rod by spread plate method. The Petri plates were incubated at 27 ± 2℃ for 24-48hrs. The isolated colonies were observed after incubation and colony characters were recorded and then subcultures on Mannitol N-free Agar. They are subjected to different biochemical tests. The purified isolates were preserved at 4^{o}C.

3.4 Identification of *A. chroococcum*

The test isolates were identified on the basis of cultural characterization, microscopic examination, pigmentation, biochemical tests and physiological test as described in Bergey's Manual of Systemic Bacteriology (Krieg et al 1994). Colony morphology, gram staining, production of diffusible and non-diffusible pigments was determined on Mannitol N-free Agar. The pigment of colonies of *Azotobacter* isolates was noted after a week on a Mannitol N-free Agar. Biochemical tests include methyl red, Voges Proskauer, indole, oxidase test, catalase test, nitrate reduction, starch hydrolysis test and cyst formation were also carried out. Finally, *A. chroococcum* was identified.

3.5 PGPR characteristics

3.5.1 Indole acetic acid production

Qualitative analysis of indole acetic acid was done by inoculating *A. chroococcum* in Luria Bertani (LB) amended with 0.5gm/l L-tryptophan and incubating at 27^{o}C in rotatory shaker at 150rpm for 2-3 days. Cell free supernatants were then prepared by centrifuging the broth at 5000rpm for 15 min at 4^{o}C. 1ml of Salkowski's reagent with 1 ml of cell free broth and 2 drops of orthophosphoric acid was added and kept in dark for 20 min in $27 \pm$

2℃ then pink color was noted qualitatively. Those isolates producing highest and lowest amount of pink color was selected for plant inoculation.

3.5.2 Hydrogen cyanide production

HCN production was detected by inoculating the bacteria on modified nutrient agar media amended with 4.4% glycine/liter according to Lorck (1948) what man filter paper no.1 previously soaked in 2%sodium carbonate in 0.5% picric acid solution was placed in the lid of the petri dish and seals with paraffin to air tight. HCN production was indicated by color orange to brownish red. The isolates producing highest amount of pink color was selected for antagonistic activity against selected phytopathogens.

3.5.3Determination of ammonia production

The test isolates were tested for the production of ammonia using the qualitative method of Ahmad et al (2008). Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 28±2°C. Nessler's reagent (0.5ml) was added in each tube. The development of a brown to yellow color was indicative of ammonia production.

3.6 Isolation of plant pathogens

The diseased plant showing typical wilt or disease symptom were collected from field and brought to the laboratory, washed rapidly with sterile water. Thereafter, small pieces of diseased portion along with healthy portion were cut using sterile blades and surface sterilized in 1% sodium hypo chloride for 5 min followed by thorough rising in sterile distilled water, three times. The surface sterilized pieces were then inoculated aseptically in a plate containing sterile PDA separately and incubated at the laboratory conditions at 27 ± 2℃ for 24-72 hrs. After 72 hours the mycelium growing the margin of diseased portion was subculture on fresh PDA plate. In this way pure culture of Phyto pathogens were isolated.

3.7 Purification and Identification of Phytopathogens Isolates of plant pathogenic fungi

The cultures of phytopathogenic fungi were purified separately by transferring the tip of the mycelia into PDA slants by hyphal tip method as described by Nelson (1982) and maintained as stock cultures for further studies. Isolated fungus was identified according to their morphological characters based on T Suneo Watanabe (2010) and stored at 4^{o}C until use.

3.8 Screening of antagonistic effect of *A. chroococcum* against phytopathogens by dual culture.

A. chroococcum was selected for antagonistic effect on the basis of HCN production *A. chroococcum* five isolates AB60, AD2, AI32, AT25 AND AJ4 were tested for their potential to antagonized three pathogens viz, Fusarium *oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii* using in vitro dual culture method.

To test the antagonistic effect of the above phytopathogens and *A. chroococcum* in vitro studies were conducted by adopting the standard methods, loopful of bacterial culture was placed (5mm in diameter)at one edge on the periphery of modified Mannitol N-free Agar media supplemented with 2%sucrose and mycelial discs (5mm in diameter) were cut from actively growing 5 days old fungus culture and placed opposite to the bacterial inoculation on plate .In control plate, only fungal pathogens were placed at one edge of Petri plate without any bacterial isolate. The assay of dual culture interaction was conducted in triplets in completely randomized design. The radius of mycelia growth in treatment and control plate was measured for 3 days.

The inhabitation percent of mycelial growth was calculated using the formula (Skidmore and Dickinson, 1976).

$$\mathrm{I} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100\%$$

Where I=Inhibition percentage (%) or antagonistic effect `Q=colony diameter of test fungus in control plate and T=colony diameter of the same test fungus in dual culture against *A. chroococcum* as antagonist. Five isolates of *A. chroococcum* were tested in vitro for their antagonist activity against test plant pathogens.

3.9 Application of *A. chroococcum* for PGPR effects

Five isolates of *A. chroococcum* one from each area were selected for inoculating on seed on the basis of indole acetic acid production.AD2, AB60, AJ18, AI35 and AT26 were selected.

3.9.1 Inoculums preparation

The pure colony of were grown in Mannitol N-free broth for 48 hrs. at 27 ± 2℃ in a rotating shaker (150rpm) and cultures were centrifuged at 10,000 for 5 min at 48°C. The culture supernatants were discarded and pellets were washed and re-suspended in SDW to obtain the final bacterial population density. the pellet was collected aseptically and Cell suspension of bacterial strains was mixed with 1ogm/l carboxymethylcellulose (CMC) solution, separately to form slurry coated on the surface of seeds, as described by Gupta et al (2002) which work as adhesion for bio-coating the seed of okra plants.

3.9.2 Biopriming of plant's seed

The method described by Weller and Cook, (1983) was adopted for seed bacterization or biopriming. Certified seeds of Durga seed farm, Chandigarh, were surface sterilized with 0.1% HgCl2 for 3 min, then rinsed with 10 changes of SDW and dried overnight under a sterile air stream. Sterilized seed of plants were then placed in bacterial CMC suspension for about 30 min. Seeds, coated with 1ogm/l CMC suspension without bacterial coating, served as the control. The bio coated seedling will be transplanted into pot.

3.9.3 Potting

Sterile soil with equal volume of compost was filled in sterile pot. The bio-coated seeds were transplanted into pot making three replicates per treatment using a completely randomized block design

Seeds coated with 10gm/l CMC suspension without bacterial coating, served as the control.

Plants were maintained in normal conditions at my garden by watering daily and equal moisture was maintained in each pot. Four seeds of okra were sown per pot. After germination, plants were thinned to one per pot. One plant per pot was selected for recording the data in all the treatments, plant height and numbers of leaves were measured at 15 days' interval for two months after transplantation (DAT). While the root length, fresh and dry weights of plants were measured after harvesting.

3.9.4 The measured parameters

a) The height of the plant (in cm):

It was measured from the rhizosphere region to the upper end of the plants.

b) Number of leaves

Total number of leaves per plant was counted and recorded.

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

It was measured lower from rhizospheic region after harvesting.

d) The fresh weight of the plant (in g)

The biomasses of the plants were weighed fresh after harvesting.

e) The dry weight of the plant (in g)

The biomass of the plants was estimated after they were carefully rinsed with water, dried with filter paper then placed at oven at 75°C until the weight was stabilized.

3.10 Data Analysis

The data recorded from dual culture and pot culture were documented and tabulated. The data were statistically analyzed using SPSS version 16. One-way ANOVA test was used to determine the association of plant growth parameters with different treatments. The test was statistically significant if $P<0.05$ with 95% Confidence interval.

FLOW CHART

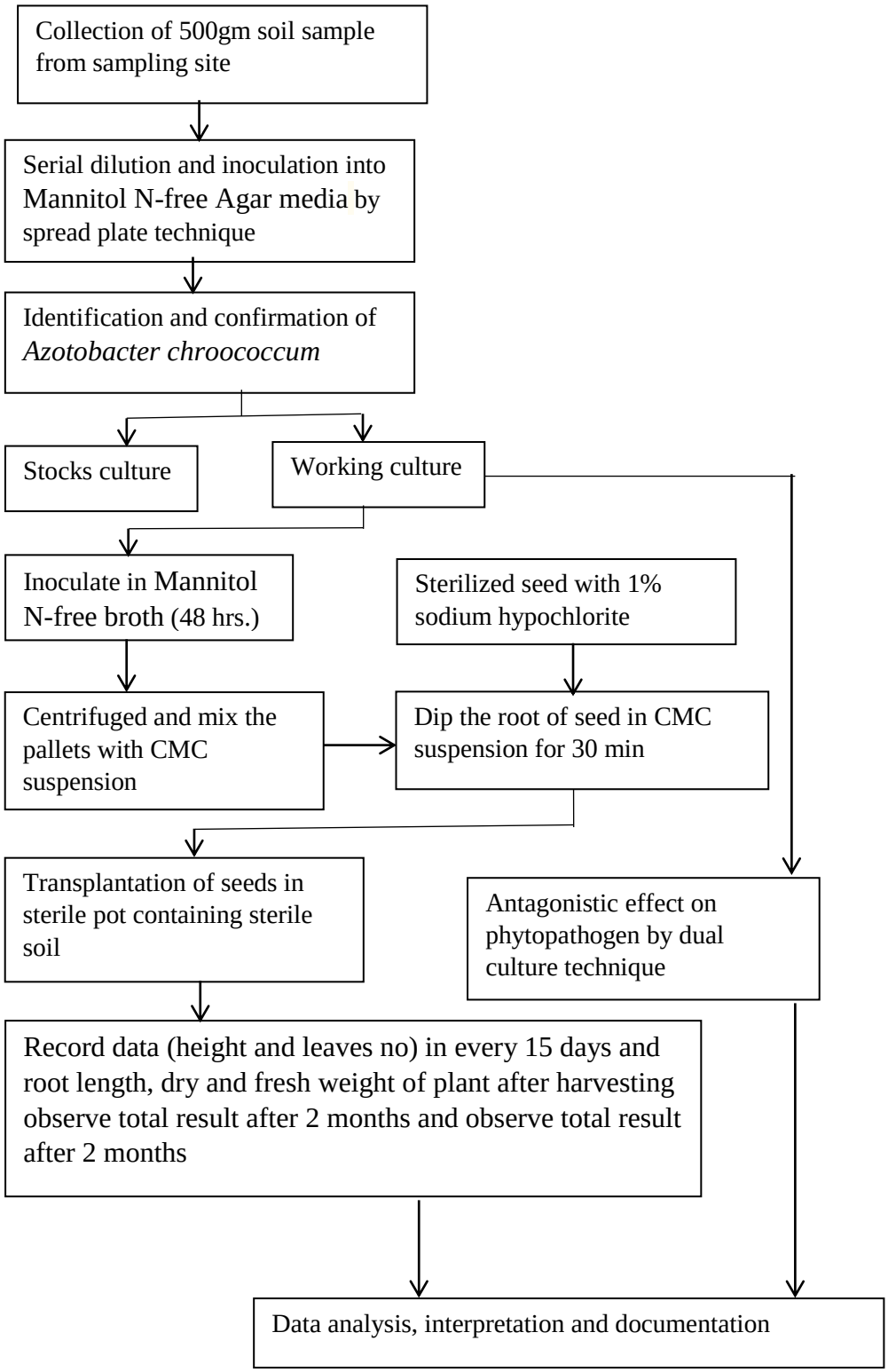


Fig.1. Schematic diagram for Antagonistic effects of *Azotobacter chroococcum* on phytopathogen and on growth of *Abelmoschus esculentus* (okra).

CHAPTER IV

RESULT

4.1 Population of *A. chroococcum*

Out of 50 soil sample collected from different agricultural areas in eastern Nepal (Itahari, Tarahara, Dharan, Jhapa, Biratnagar).10 samples were collected from each area. *A. chroococcum* were isolated from 30 samples by serial dilution technique using *Azotobacter* agar (mannitol)media. 60% samples were found to be positive for *A. chroococcum.* (Fig.4.1)

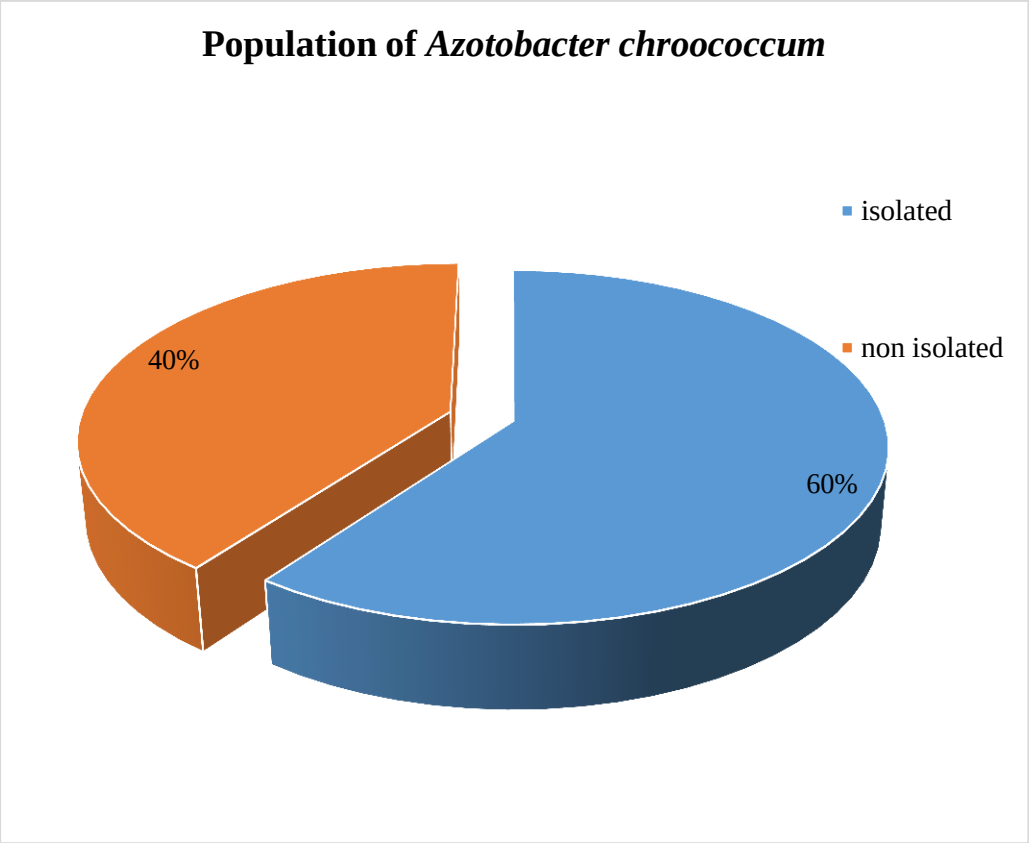


Fig.4.1 Population of *A. chroococcum* from 50 soil samples collected.

4.2 Percentage occurrence of isolated population of *A. chroococcum*

Out of 30 samples isolated from the study area.7, 4,4,5 and 12samples were isolated from Itahari, Biratnagar, Jhapa, Dharan and Tarahara respectively. The samples were collected from rhizospheric soil of different crops (tomato, maize, beans, peas, potato, paddy, mustard, pumpkin, cabbage, cauli, brinjal, sugarcane, garlic, Spanish, chilly and wheat) (Fig 4.2).

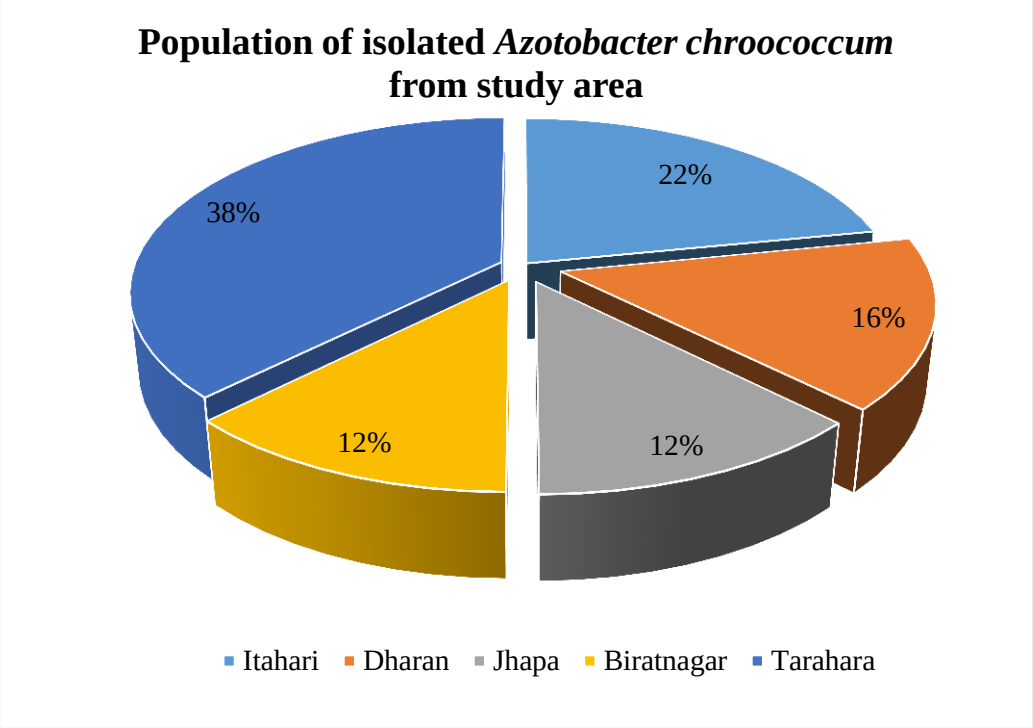


Fig 4.2: Percentage occurrence of isolated population of *A. chroococcum* from study area.

4.3 Morphology and Biochemical characteristics of *A. chroococcum.*

Among 30 isolates of *A. chroococcum* strains were identified in soil samples collected from the rhizosphere. These strains were purified and characterized thorough microscopically and biochemical test cell shape, pigmentation, colony size, Gram reaction and catalase activity. Microscopic and macroscopic examinations of the Gram-negative bacilli, capable to form cyst, white, transparent, viscous and moist colonies which turn dark brown after 5-7 days of incubation on a mannitol N-free agar medium along with the biochemical tests revealed the identity of different *A. chroococcum* strains. Bacterial isolates were tested for the production of ammonia in peptone water. Isolates was positive for ammonia production by development of brown to yellow color are shown below in table 4.1

Table. 4.1: Morphology and Biochemical characteristics of *A. chroococcum.*

Characteristics	***Azotobacter chroococcum***
Gram staining	Gram Negative
Shape	Small rod, single cocci, coccoidal chain, cocci in clumps
Color	Milky, glistening, gummy with dull to cream white
Consistency	Transparent, convex, viscous moist colonies
Margin	Circular, raised, smooth
Cyst formation	+
Mortality	+
Nitrate reduction	+
Black brown pigmentation	+
Starch hydrolysis	+
Catalase	+
Oxidase	+
Citrate utilization	+
Ammonia production	+

4.4 Indole acetic acid and HCN production

Isolates were tested for the indole acetic acid production qualitatively on the basis of pink color production by cell free supernatant after addition of orthophosphoric acid. Out of 30 isolates,24 isolates were positive for IAA production and 6 isolates were found not determined.

Similarly, hydrogen cyanide was quantified on the basis of conversion of yellow colored what's man filter paper soaked in 2% sodium carbonate in 0.5% picric acid into brownish red. Out of 30 isolates, 28 isolates were positive for HCN production and 2 isolates were found not determined are shown below in table 4.2

Table 4.2 Indole acetic acid and HCN production

SN	Positive production	Not determined
HCN	28	2
IAA	24	6

4.5 Isolation of plant pathogens

Among 3 pathogenic fungi were isolated from different types of diseased parts of plants by tissue culture techniques on PDA and CMA then identified by LPCB staining. Diseased plants and their respective pathogenic fungi isolated are shown below in table 4.3

Table 4.3 Disease plants and their respective phytopathogens

S.N.	Plants	Plant's Part	Organism	Disease
1	Banana	Leaf	*Fusarium oxysporum*	Banana fusarium wilt
2	Rice	Steam	*Rhizoctonia. solani*	Sheath Blight
3	Rice	Stem and root	*Sclerotium rolfsii*	Seedling blight

4.6 Antagonistic effect of *A. chroococcum* against phytopathogens

Antagonistic activities of five isolates AB60, AD2, AI32, AT25 and AJ4 were observed by inoculating the loopful bacterial culture at one edge on periphery of modified Mannitol N-free Agar media supplemented with 2%sucrose and mycelial discs of actively growing 5 days old fungal culture was placed opposite to the bacterial inoculation on plate. In control plate, only fungal pathogens were placed at one edge of Petri plate without any bacterial isolate. The assays were conducted in triplicates in completely randomized design. The radius of mycelia growth in treatment and control plate was measured for 3 days.

4.6.1 Antagonistic effect of *A. chroococcum* against *F. oxysporum*

Out of chosen 5 isolates. IP value of AD was significantly higher followed by AT, AB, AJ and AI respectively in day 4 and day 5 whereas there was no significant difference in day 3. Isolates AD inhibited *F. oxysporum* by 24.9% and found to be potent in controlling *F. oxysporum* are shown below in table 4.4.

Table 4.4 Antagonistic effects of *A. chroococcum* isolates against *F. oxysporum*

SN	Isolates	Inhibition percentage (IP in %)			
		Day3	Day4	Day5	Average
1	AD	30.2	29.1[a]	15.4[a]	24.9%
2	AB	18.6	27.5[b]	10.3[c]	18.8%
3	AT	26	27.5[b]	11.2[b]	21.57%
4	AJ	21.7	18.1[c]	10.3[c]	16.70%
5	AI	18.6	13.3[d]	9.3[d]	13.73%
6	p-value	.194	.039	.011	

Note: mean of three replications. Different letter in each column denotes significant differences (P<0.05) among the treatments and same letters

followed in the columns are not significantly different according to Duncan's multiple range test.

4.6.2 Antagonistic effects of *A. chroococcum* isolates against *Sclerotium rolfsii*

Out of chosen 5 isolates. IP value of AB was significantly higher followed by AI, AT, AJ and AD respectively in day 3 and day 5 whereas there was no significant difference in day 4. Isolates AB inhibited *S. rolfsii* by 35.5% and found to be potent in controlling *S. rolfsii* are shown below in table 4. 5.

Table 4.5 Antagonistic effects of *A. chroococcum* isolates against *S. rolfsii.*

SN	Isolates	Inhibition percentage (IP in %)			
		Day3	Day4	Day5	Average
1	AD	23.8^{d}	13.2	16^{e}	17.6%
2	AB	34.3^{a}	39.8	26.4^{a}	35.5%
3	AT	25.3^{c}	12.5	19.2^{b}	20.06%
4	AJ	25.3^{c}	9.5	17.6^{d}	18%
5	AI	26.8^{b}	25.7	22.4^{b}	23.37%
6	p-value	.004	.146	.011	

Note: mean of three replications. Different letter in each column denotes significant differences ($P<0.05$) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, AD= *A. chroococcum* from Dharan, AB== *A. chroococcum* from Biratnagar, AT== *A. chroococcum* from Tarahara, AJ== *A. chroococcum* from Jhapa, AI== *A. chroococcum* from Itahari

4.6.3 Antagonistic effects of *A. chroococcum* isolates against *Rhizoctonia solani*

Out of chosen 5 isolates. IP value of AD was significantly higher followed by AB, AT, AJ and AI respectively in day 3 and day 5 whereas there was no significant difference in day 4. Isolates AB inhibited *R. solani* by 47.8% and found to be potent in controlling *R. solani* are shown below in table 4.6

Table 4.6 Antagonistic effects of *A. chrooocoocum* isolates against *R. solani*

SN	Isolates	Inhibition percentage (IP in %)			
		Day3	Day4	Day5	Average
1	AD	34.9[a]	42.5	45[a]	47.8%
2	AB	26.9[b]	31.5	40[b]	36.4%
3	AT	26.9[b]	48	35[c]	34.80%
4	AJ	25.3[c]	42.5	30[d]	34.4%
5	AI	25.3[c]	63.7	23.3[e]	26.7%
6	p-value	.003	.24	.004	

Note: mean of three replications Different letter in each column denote significant differences (P<0.05) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, AD = *A. chroococcum* from Dharan, AB== *A. chroococcum* from Biratnagar, AT== *A. chroococcum* from Tarahara, AJ== *A. chroococcum* from Jhapa, AI== *A. chroococcum* from Itahari

4. 7 In vitro effects of *A. chroococcum* isolates on *Abelmoschus esculentus* (okra) growth

Five *A. chroococcum* isolates (one from each district) were subjected to in-vivo PGPR effect on okra plant. Isolates were selected on the basis of indole acetic acid production. Height and number of leaves were measured at 15 days' interval for 60 days where root length, fresh weight and dry weight were measured after harvest of plants on 60 days of inoculation. then data were compared with the control where no isolates were inoculated. AB60 from Biratnagar (T1), AD2 from Dharan (T2), AJ1 from Jhapa (T3), AI35 from Itahari (T4), AT26 from Tarahara(T5) were different isolates used and three replicates were maintained each isolate along one plant per pot.

All the treatments of *A. chroococcum* were compared to the control. All the parameters related to the plant growth and yields were also maximum in treated plant than control plant.

4.7.1 Effects of *A. chroococcum* isolates on number of leaves of *Abelmoschus esculentus* (okra).

After 15 DAT of seedlings to the pot, the number of leaves of plant was recorded at the interval of 15, 30, 30 45 and 60 days. As seen in table below, there was no significant difference in number of leaves in plants between the treatments and the control plants till the day of harvesting in (60days).

Table 4.7 Effects of *A. chroococcum* isolates on number of leaves of plant

SN	Isolates	leaves number			
		Day15	Day30	Day45	Day60
1	Control	3	5	7	8
2	T1	3.67	6.3	7.33	9
3	T2	4	6.33	7.67	8.673
4	T3	3.67	6	7.67	8.33
5	T4	3.33	5.33	7.33	8.33
6	T5	3.33	4.3	6.66	8
p-value		.123	.524	.503	.123

Result: $p<0.05$. There was significant difference in numbers of leaves among plants within each treatment groups.

Note: mean of 3 replications. Different letter in each column denotes significant differences ($P<0.05$) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, DAT = Days after transplantation. T1 = AB60 (Biratnagar), T2 = AD2 (Dharan), T3 = AJ18 (Jhapa), T4 = AI35 (Itahari), T5 = AT26 (Tarahara).

4.7.2 Effects of *A. chroococcum* isolates on height of plant.

After 15 DAT of seedlings to the pot, plant height (cm) was recorded at the interval of 15, 30, 45 and 60 days. All the treatments are compared to the control (Table 4.5). All the parameters related to the plant growth and yields were also maximum in treatment compared to control. The height of plants treated with T2 and T3 were significantly higher than T1, T4, T5 and control plants in day 30 and day 45 and whereas no significant difference was found in day 15 and day 60. These isolates may produce growth promoting phytohormones like indole acetic acid (IAA), or auxin analogues and vitamins that supports plant growth. are shown below in table 4.8.

Table 4.8 Effects of *A. chroococcum* isolates on height of plant

SN	Isolates	Height in cm			
		Day15	Day30	Day45	Day60
1	Control	11	19[c]	25[d]	33
2	T1	13	22.33[a]	27.67[c]	39.33
3	T2	13.67	23[a]	38.67[a]	56.33
4	T3	13.67	22.33[a]	38[a]	55.33
5	T4	12.67	22[b]	26.5[c]	40.5
6	T5	13.3	22[b]	33[b]	39.3
p-value		.270	.009	.002	.278

Result: $p<0.05$. There was significant difference in plant height among plants within each treatment groups.

Note: mean of 3 replications. Different letter in each column denotes significant differences ($P<0.05$) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, DAT = Days after transplantation. T1 = AB60 (Biratnagar), T2 = AD2 (Dharan), T3 = AJ18 (Jhapa), T4 = AI35 (Itahari), T5 = AT26 (Tarahara).

4.7.3 Effects of *A. chroococcum* isolates on root length of plant.

After harvesting the plant at 60 days the root length of the plants was measured and tabulated. The root length of the plant treated with T4 significantly higher than other isolates as well as than control plants. Root length of all the treatments were significantly higher than control plants. T2 was at par with T3. The maximum root length was measured for treatment T4 and the minimum was for control.

Table 4.9 Mean root length (cm) of control and treated plants.

SN	Isolates	root length (cm)	P-value
1.	Control	8.5^{e}	0.48
2.	T1	11.17^{c}	
3	T2	12.17^{b}	
4.	T3	12.29^{b}	
5.	T4	13.05^{a}	
6.	T5	10.25^{d}	

Result: $p<0.05$. There was significant difference in root length among plants within each treatment groups.

Note: mean of 3 replications. Different letter in each column denotes significant differences ($P<0.05$) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, T1 = AB60 (Biratnagar), T2 = AD2 (Dharan), T3 = A J18 (Jhapa), T4 = AI35 (Itahari), T5 = AT26 (Tarahara).

4.7.4 Effects of *A. chroococcum* on Fresh weight of plant.

During the experiment, the weight of fresh weight of plant was also measured at 60 days. The fresh weight of plant treated with T2 isolates was significantly higher than other isolates as well as control plants. There was difference in fresh weight between five treatments and control plants. The maximum weight was measured for treatment T2 and the minimum was for control.

Table 4.10 Mean fresh weight (g) of control and treated plants.

SN	Isolates	Fresh weight(g)	P-value
1.	Control	7.57^{e}	.024
2.	T1	13.55^{c}	
3	T2	36.34^{a}	
4.	T3	35.41^{b}	
5.	T4	12.95^{d}	
6.	T5	14.5^{c}	

Result: $p<0.05$. There was significant difference in fresh weight among plants within each treatment groups

Note: mean of 3 replications. Different letter in each column denotes significant differences ($P<0.05$) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, T1 = AB60 (Biratnagar), T2 = AD2 (Dharan), T3 = AJ18 (Jhapa), T4 = AI35 (Itahari), T5 = AT26 (Tarahara).

4.7.5 Effects of *A. chroococcum* on dry weight of plant.

After the analysis fresh weight of plants, the plants were washed properly, air dried on filter paper and then placed at oven at 75°C until the weight was stabilized. Dried plants were measured (g) and tabulated. The dry weight of plant treated with T2 isolates was significantly higher than other isolates as well as control plants followed by T3, T5T1, T4 and T5 respectively. There was no difference in fresh weight between T1 treatment T5. The maximum weight was measured for treatment T2 and the minimum was for control.

Table 4.11 Mean Dry weight (g) of control and treated plants.

SN	Isolates	Dry weight(g)	P-value
1.	Control	2.93^{d}	0.05
2.	T1	6.79^{c}	
3	T2	11.34^{a}	
4.	T3	6.79^{c}	
5.	T4	8.4^{b}	
6.	T5	11.11^{a}	

Result: $p<0.05$. There was significant difference in dry weight among plants within each treatment groups.

Note: mean of 3 replications. Different letter in each column denotes significant differences ($P<0.05$) among the treatments and same letters followed in the columns are not significantly different according to Duncan's multiple range test, T1 = AB60 (Biratnagar), T2 = AD2 (Dharan), T3 = AJ18 (Jhapa), T4 = AI35 (Itahari), T5 = AT26 (Tarahara).

CHAPTER V
DISSCUSSION

The present investigation deals with the Growth promotional effect of *A. chroococcum* on plant (Okra) and its antagonistic activity against some selected phytopathogens and the result obtained from the investigation have been critically and discussed here with appropriate interpretation, facts and comparison with previous works are mention in this chapter v.

In this study different isolates of *A. chroococcum* were isolated from 5 different places of Eastern Nepal and these isolates were subjected to dual culture method against different phytopathogens and seed treatment was done to observe the growth promotional effect (PGPR) of isolates on okra plant.

The species of *Azotobacter* are dominant in rhizosphere among the nitrogen-fixing bacteria. These are Gram negative, motile, cyst former rods with acuminated ends. There are many known factors that contribute to the PGP activities of *Azotobacter* such as N2 fixation, IAA production, phosphate solubilization (Kumar and Narula, 1999), siderophore production (Fekete et al. 1989; Verma et al. 2001), ACC deaminase activity, antagonism towards various plant pathogens (Joshi et al 2006) and lytic enzymes (Gupta et al. 1999; Singh et al 2008). A bacterium can affect plant growth by one or more of these mechanisms and uses different abilities for growth promotion at various times during the life cycle of plants.

Many studies have been conducted to observe the antagonistic activity of rhizospheric microorganisms. Production of antifungal substances by soil isolates of *A. chroococcum* is known since last many years. Brown and Burlingham, (1983) emphasized the antimicrobial activity of *Azotobacter* spp. in relation to Phyto pathogenicity. Antifungal activity was also detected in *A. chroococcum* by Lakshmi Kumari et al (1972) and Sharma et al (1986). Suppressive effect of *A. chroococcum* on *Rhizoctonia solani* was studied by Meshram and Jager, (1983) and Meshram, (1984). There would have been a key role of diffusible compounds of bacteria that caused the mycelial and sclerotia degeneration. It may be due to the production of antimicrobial

substances such as chitinolytic enzyme, antibiotic substances, siderophore and nutrient competition as described above. Involvement of secondary metabolites, competition for nutrients and space, colonization, physical and chemical factors, etc. may also have been involved during antagonism (Fray 2002).

The present study showed the in-vitro potential of five isolates of *A. chroococcum*. This may be production of antibiotic production of *A. chroococcum* in medium. In my research *A. chroococcum* isolates showed potential in controlling *R. solani* in–vitro but with varying degrees of inhibition amongst the isolates. Biocontrol order of potential (BOP) for *R. solani* was found to be AD≥AB≥AT≥AJ≥AI.AD suppress the growth by 47.8% at days 5 on inoculation followed 36.4%, 34.80%, 34.4% and 27.7% by AB, AT, AJ, AI respectively AI being least potent with IP Value 27.7%. While the control plates showed regular radial growth covering the whole plates on 5 days. Antagonistic effects were more pounced by 3 days after inoculation. This result was supported by Verma et al (2001) demonstrated the in vitro production of antimicrobial/antifungal substances by different strains of *A. chroococcum*. They found that only 37% of the total strains were able to inhibit the growth of *R. solani*. Similar research was also conducted by Alsudani et al (2020). It was observed that *A. chroococcum* showed the strongest antagonistic activity followed by *P. fluorescens* with the percentage of inhibition ranging between (72.9-77.1) and (69.5-70.3) % for *R. solani* and *F. solani* respectively after 7 days of incubation.

Biocontrol order of potential (BOP) for *F. oxysporum* was found to be AD≥AT≥AB≥AJ≥AI.AD had the highest IP value 24.9% at days 5 on inoculation followed 21.57% ,18.3%,16.70% and 13.73% by AT, AB, AJ, AI respectively.AI being least potent with IP Value 13.73%. This result was supported by Chauhana et al (2011) found that *A. chroococcum* were found to be effective biocontrol agents against *R. solani* cotton (disease index 16.7%) and *R. solani* rice (2.5%) in cotton crop, whereas in guar, the crop disease index was 2.5% against *R. solani* cotton, 13.3% against *R. solani* rice and 10% against *F. oxysporum* tomato in tomato crop compared with their respective checks.

The observations recorded in the present investigation are in conformity with the findings of Maiyappan et al (2010), who found that the *F. oxysporum* growth was effectively minimized by *Azotobacter* strain 5 (49.84%) followed by *Azotobacter* strain 9 (49.04%). Kapoor and Kar (1989) reported the antagonistic activity of *A. chroococcum* against *F. oxysporum*. The *A. chroococcum* strains showing inhibition by 11.7 to 24.7 per cent after 7 days.

Biocontrol order of potential (BOP) for *S. rolfsii* was found to be AB≥AI≥AT≥AJ≥AD.AB suppress the growth by 35.5% at days 5 on inoculation followed 23.37%, 20.06%, 18% and 17.6% by AI, AT, AJ, AD respectively AD being least potent with IP Value 17.6%. These observations are in agreement with the findings of earlier workers Maareg et al (2003), who reported that the substantial control of *S. rolfsii* by microbin was observed on clay loam soils (74%) and also Maiyappan et al (2010) found that the *Azotobacter* 7 strain recorded maximum inhibition against (54.04%) followed by *Azotobacter* strain 6 with (43.18%). Also, similar studies conducted by Patil et al (2014) antagonist studies, AZ 8 isolate was found significantly superior for control of *R. solani* AZ 21 isolate for *S. rolfsii* and AZ 1 for *F. oxysporum* f. sp. *cicer*

The results showed that the *A. chroococcum* can play an important role in biocontrol of soil borne diseases of rhizosphere. Although in vitro assay does not always provide the reliable data on potential of biocontrol agents, they provide guidelines to apply them in vivo assay for controlling the phytopathogens (Chaves et al 1991)

Effects of 5 different isolates of *A. chroococcum* (AD, AB, AJ, AI, AT) on growth of okra plant has been observed in present study found that there was high significant difference in height of okra plant between treated and control plants. Number of leaves, plant height, root length, fresh and dry weight of per plant found that the maximum was recorded in the treatment.

Among five isolates T2 isolates has the highest effect on plant height(56.33cm) followed by T3, T4, T1 and T5 with height 55.33cm, 40.5 cm, 39.33cm and 39.3cm respectively whereas control plant has only 33cm height in 60 days. There was no significant difference in number of leaves

between treated plant and control plant in during harvesting time (60 days). Similarly, T4 isolates has highest root length (12.95cm) whereas control plant has only 8.5cm root length after harvesting. Fresh weight of T2 has the highest weight (36.34gm) whereas control plant has 7.57gm after harvesting. Also, the dry weight of plants after drying in oven and T2 has highest weight (11.34gm) compared to control plant which is observed as 2.93 after drying.

The result obtained was supported by Salhia (2013) *Azotobacter* inoculants have a significant promoting effect on growth parameters like root, shoot length and dry mass of bamboo and maize seedlings in vitro and in pot experiments. Similar research was conducted by Estiyar et al (2014) reported that, number of branches, pod per plant and 1000 grain weight also increased with *Azotobacter* application. Similar result put forwarded by Sandeep et al (2011) which revealed that there is better growth response of *Azotobacter* inoculated plants as compared to non-inoculated control plants. Also result obtained by Dubey et al (2012) support the present result reported that *A. chroococcum* AZO2 strain isolated from sesame showed PGP attributes and strong antagonistic properties along with significant enhancement in crop yield the vegetative plant parameters like length, fresh and dry weight of root along with shoot length and weight (fresh and dry), after 120 days of growth period, were maximum in the treatment compared to control.

CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Biological control is an efficient and environmentally friendly way to prevent plant disease. Biological control agents have emerged as new strategies of managing plant disease by inducing systematic resistance and growth promotion of plants against diseases.

From the present study, it can be concluded that *A. chroococccum* can be serve as good option as plant growth promotor as well as its antagonistic activity serve as biocontrol against phytopathogenic fungi.

The results in this study support the potential of *A. chroococccum* as suppressor of pathogen growth in in-vitro condition and as growth promoter in in-vitro and in-vivo condition. *A. chroococccum* increased plant growths parameters such as shoot length, numbers of leaves, root length, fresh and dry weight in okra plant.

In vitro experiment was conducted to observe antagonistic effect in dual culture and from that fact it can be concluded that different isolates have different potency and potency of same isolates varied with the plant pathogens.

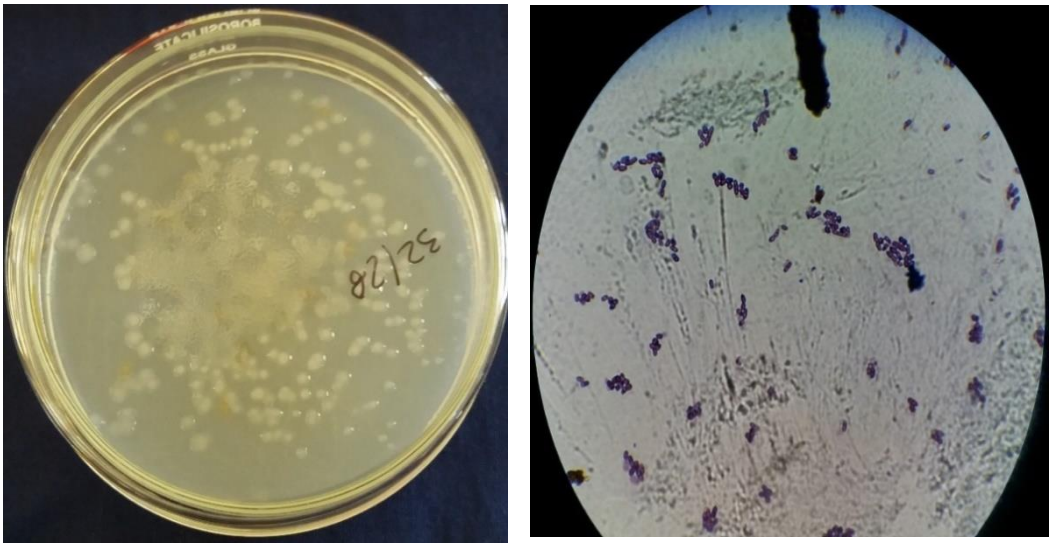
However, further studies on the effect of these treatment in field condition need to be undertaken so could be recommended as a biocontrol agent.

Thus, the finding of present investigation holds a good promise *A. chroococccum* as bio-inoculants and biocontrol agents of plant disease might be exploited for sustainable disease management programs to save the environmental risk and can be used as bio fertilizer replacing the chemical fertilizer.

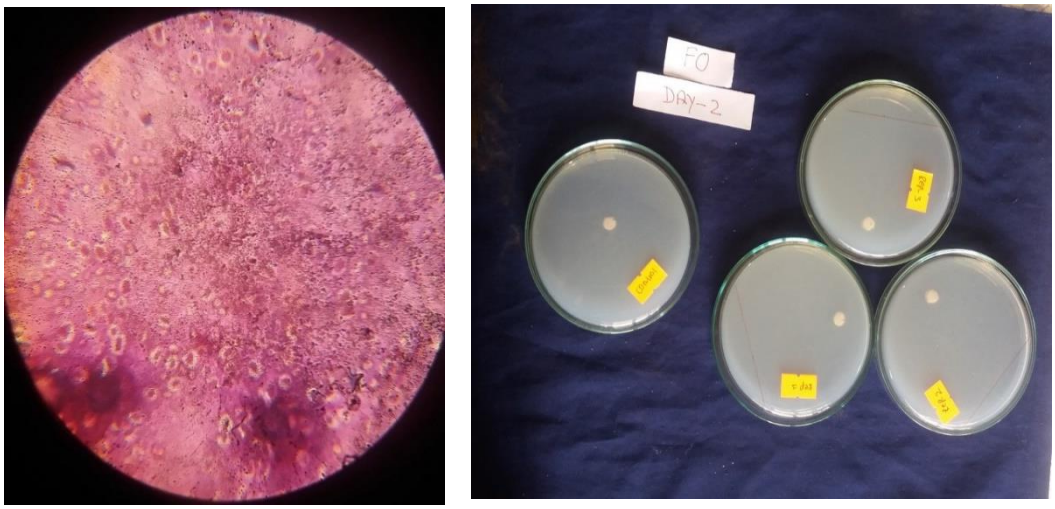
6.2 Recommendation

Based on the results and findings of the experiments, the recommendations made are as follows**:**

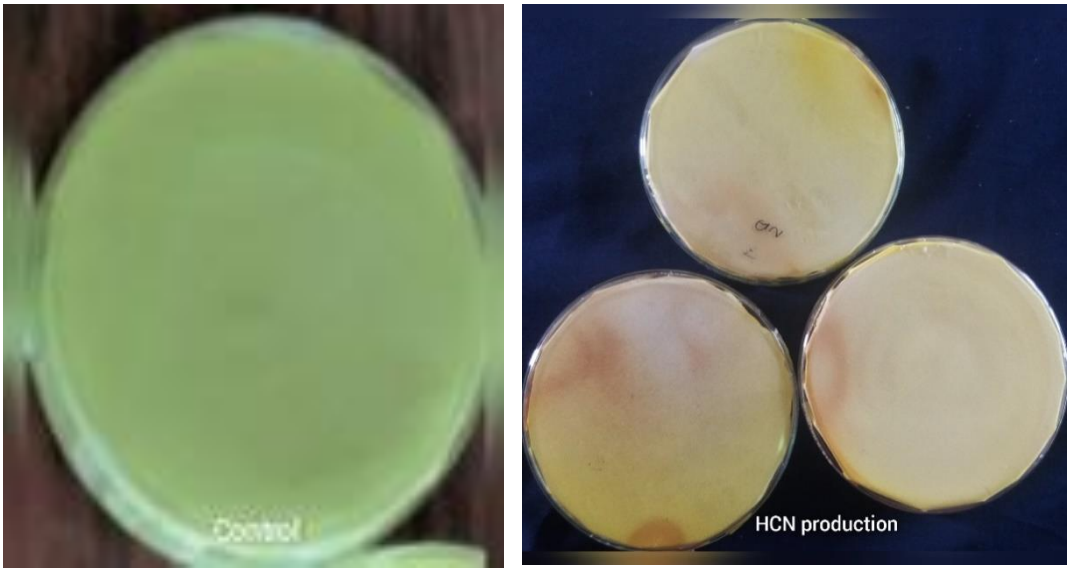
- Growth promotion results shows *Azotobacter* can be recommended as one of the best options to be used as bio fertilizer for eco-friendly and sustainable crop production due to its ability for nitrogen fixation, growth hormone production, phosphate solubilization, plant disease management and reclamation of better soil health.
- *Azotobacter* can be recommended as non-chemical means of plant disease control that can be used to protect crops from soil-borne pathogens.
- The degree of inhibition shown by *Azotobacter* against phytopathogens from this farmer can be recommended to use *Azotobacter* in place of chemicals pesticides.
- *Azotobacter* as PGPR promotes plants growth by secreting many different types of plant growth promoters like indole acetic acid, gibberellic acid. So can be recommended to use as bio fertilizer.
- In present, people are looking for organically grown product. *Azotobacter* can be recommended for mass production and awareness for use as bio pesticides and bio fertilizers.
- The degree of inhibition is least or sensitive against some phytopathogens. Thus, *Azotobacter* can be recommended to use in combination with chemical pesticides.



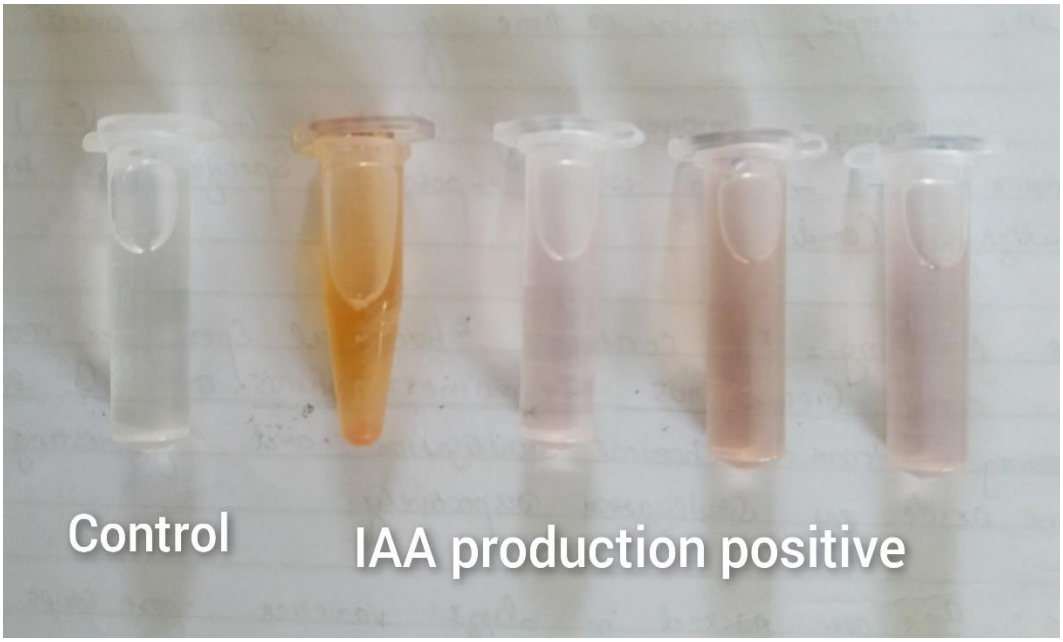
Photograph 1: Morphological and microscopic view of *Azotobacter chroococcum* respectively



Photograph 2: Cyst staining and dual culture for antagonistic effect against *Fusarium oxysporum* respectively



Photograph 3: In vitro screening of HCN production



Photograph 4: Qualitative analysis of IAA production

Photograph 5: Researcher working in lab

Photograph 6: In vitro trial (pot culture)

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APPENDIX-I

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
Laminar flow
Centrifuge
Microscope
Water bath shaker
Incubator
Digital balance

4. Reagents/stains

Safranin
Alcohol
Nessler's reagent
Lysol
Crystal violet
Bromothymol Blue

5. Culture media

Agar powder
Beef extract
Nutrient broth
Laurie Bertani broth
Peptone
Nutrient agar
Tryptone

Biochemical media

Glucose
Sucrose
Fructose
Simmons's citrate agar

APPENDIX-II

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. Potato Dextrose Agar (PDA)

Ingredients	Gms/Litre
Potatoes, infusion form	200.00
Dextrose	20.00
Agar	15.00
pH	5.6 ± 0.2

Hi media containing this entire ingredient was used. 39gof PDA base was taken in a conical flask and suspended in 1000ml distilled water. The mixture was mixed thoroughly by stirring with glass rod to have homogenized mixture. After few minutes of boiling, the pH of the medium was adjusted to 7.0 ± 0.2 and autoclaved at 15lbsp.s.i. at 120^{o}C for 20mins. To enhanced the media more potent 25.0mg of chloramphenicol was added after autoclaving when the media was cooled to temperature 40-45^{o}C.

3. Nutrient agar

Ingredient	Gms/Litre
Beef extract	0.5g
Yeast extract	1g

Peptone	2.5g
Distilled water	500mL

5. Laurie Bertani broth

Ingredients	**Gms/Litre**
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Final pH (at 25°C)	6.8± 0.2

6. Peptone broth

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

7. 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-III

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

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

Position	***Fusarium***	***Rhizoctonia***	***Sclerotium***
Kingdom	Fungi	Fungi	Fungi
Division	Ascomycota	Basidiomycota	Ascomycota
Class	Sordariomycetes	Agaricomycetes	Pezizomycotina
Order	Hypocreales	Cantharellales	Dothideomycetes
Family	Nectriaceae	Ceratobasidiaceae	Pleosporomycetidae
Genus	*Fusarium*	*Rhizoctonia*	*Sclerotium*
Species	*F. oxysporum*	*Rhizoctonia solani*	*Sclerotium rolfsii*

APPENDIX: IV

Statistical Analysis Output

ANOVA

Dual culture of *A. chroococcum* against *R. solani*

RZ (D3)

inhibition(cm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.026	1	.026	40.974	.003
Within Groups	.003	4	.001		
Total	.028	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *R. solani*

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *R. solani*.

Result: P< 0.05, so result is statistically significant

Conclusion: *A. chroococcum* isolates exhibited better inhibition against *R. solani* culture.

ANOVA

RZ (D4)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.280	1	.280	12.543	.24
Within Groups	.089	4	.022		
Total	.370	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *R. solani*

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *R. solani*.

Result: P>0.05, so result is not statistically significant

Conclusion: *A. chroococcum* isolates doesn't exhibited better inhibition against *R. solani* culture.

ANOVA

RZ (D5)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.456	1	.456	35.103	.004
Within Groups	.052	4	.013		
Total	.508	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *R. solani*

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *R. solani.*

Result: P< 0.05, so result is statistically significant

Conclusion: *A. chroococcum* (AB) exhibited better inhibition against *R. solani* culture.

ANOVA

Dual culture of *A. chroocooccum* against *Fusarium oxysporum*

FO (D3)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	2.424	.194
Within Groups	.002	4	.001		
Total	.004	5			

H_0= There is no statistical difference in dual culture inhibition of *A. choorococcum* against *F. oxysporum.*

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *F. oxysporum.*

Result: P>0.05, so result is not statistically significant

Conclusion: *A. chroococcum* isolates doesn't not exhibited better inhibition against *F. oxysporum* culture.

ANOVA

FO (D4)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.072	1	.072	9.141	.039
Within Groups	.032	4	.008		
Total	.104	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *F. oxysporum*.

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *F. oxysporum*.

Result: P< 0.05, so result is statistically significant

Conclusion: *A. chroococcum* (AD) exhibited better inhibition against *F. oxysporum* culture.

ANOVA

FO (D5)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.050	1	.050	19.610	.011
Within Groups	.010	4	.003		
Total	.060	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *F. oxysporum*.

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *F. oxysporum*.

Result: P< 0.05, so result is statistically significant

Conclusion: *A. chroococcum* (AD) exhibited better inhibition against *F. oxysporum* culture.

ANOVA

Dual culture of *A. chroocooccum* against *Sclerotium rolfsii*

SR (D3)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.028	1	.028	35.848	.004
Within Groups	.003	4	.001		
Total	.031	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *S. rolfsii*.

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *S. rolfsii*.

Result: P< 0.05, so result is statistically significant

Conclusion: *A. chroococcum* (AB) exhibited better inhibition against *S. rolfsii* culture

ANOVA

SR (D4)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.067	1	.067	3.250	.146
Within Groups	.083	4	.021		
Total	.150	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *S. rolfsii*.

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *S. rolfsii*.

Result: P>0.05, so result is not statistically significant

Conclusion: *A. chroococcum* isolates doesn't exhibited better inhibition against *S. rolfsii* culture.

ANOVA

SR (D5)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.215	1	.215	20.061	.011
Within Groups	.043	4	.011		
Total	.258	5			

H_0= There is no statistical difference in dual culture inhibition of *A. chroococcum* against *S. rolfsii*

H_1=There is statistical difference in dual culture inhibition of *A. chroococcum* against *S. rolfsii*

Result: P< 0.05, so result is statistically significant

Conclusion: *A. chroococcum* (AB) exhibited better inhibition against *S. rolfsii* culture.

ANOVA

Growth parameters

Leaves number (Day15)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.300	1	.300	3.802	.123
Within Groups	.316	4	.079		
Total	.616	5			

H_0= There is no statistical difference in leaves yield of plants with different treatment groups in day15.

H_1=There is statistical difference in leaves of plants with different treatment groups in day15.

Result: P>0.05, so result is not statistically significant

Conclusion: Treatments doesn't exhibit better growth of plant leaves than control and other treatment groups in day15.

ANOVA

Leaves number (Day30)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.361	1	.361	.486	.524
Within Groups	2.972	4	.743		
Total	3.333	5			

H_0= There is no statistical difference in leaves yield of plants with different treatment groups in day30.

H_1=There is statistical difference in leaves of plants with different treatment groups in day30.

Result: P> 0.05, so result is not statistically significant

Conclusion: Treatments doesn't exhibit better growth of plant leaves than control and other treatment groups in day30.

ANOVA

Leaves number (Day45)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.092	1	.092	.540	.503
Within Groups	.680	4	.170		
Total	.772	5			

H_0= There is no statistical difference in leaves yield of plants with different treatment groups in day45.

H_1=There is statistical difference in leaves of plants with different treatment groups in day45.

Result: P>0.05, so result is not statistically significant

Conclusion: Treatments doesn't exhibit better growth of plant leaves than control and other treatment groups in day 45.

ANOVA

Leaves number (Day60)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.238	1	.238	1.636	.123
Within Groups	.581	4	.145		
Total	.819	5			

H_0= There is no statistical difference in leaves yield of plants with different treatment groups in day60.

H_1=There is statistical difference in leaves of plants with different treatment groups in day60.

Result: P>0.05, so result is not statistically significant

Conclusion: Treatments doesn't exhibit better growth of plant leaves than control and other treatment groups in day 60.

ANOVA

Plant height (Day15)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.264	1	4.264	22.636	.270
Within Groups	.753	4	.188		
Total	5.017	5			

H_0= There is no statistical difference in height of plants with different treatment groups in day15.

H_1=There is statistical difference in height of plants with different treatment groups in day15.

Result: P>0.05, so result is not statistically significant

Conclusion: Treatments doesn't exhibit better growth of plant than control and other treatment groups in day 15.

ANOVA

Plant height (Day30)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.252	1	9.252	55.510	.009
Within Groups	.667	4	.167		
Total	9.919	5			

H_0= There is no statistical difference in height of plants with different treatment groups in day30.

H_1=There is statistical difference in height of plants with different treatment groups in day30.

Result: P<0.05, so result is statistically significant

Conclusion: T2=AD2(Dharan)treatment exhibited better growth of plant than control and other treatment groups in day 30.

ANOVA

Plant height (Day45)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	50.285	1	50.285	1.577	.002
Within Groups	127.539	4	31.885		
Total	177.824	5			

H_0= There is no statistical difference in height of plants with different treatment groups in day45.

H_1=There is statistical difference in height of plants with different treatment groups in day45.

Result: P<0.05, so result is statistically significant

Conclusion: T2=AD2(Dharan)treatment exhibited better growth of plant than control and other treatment groups in day 45.

ANOVA

Plant height (Day60)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	144.277	1	144.277	1.842	.278
Within Groups	313.262	4	78.315		
Total	457.539	5			

H_0= There is no statistical difference in height of plants with different treatment groups in day60

H_1=There is statistical difference in height of plants with different treatment groups in day60

Result: P>0.05, so result is not statistically significant

Conclusion: Treatments doesn't exhibit better growth of plant than control and other treatment groups in day 60.

ANOVA

Root length (Day60)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.780	1	8.780	7.989	0.48
Within Groups	4.396	4	1.099		
Total	13.177	5			

P=0.48

H_0= There is no statistical difference in fresh root weight of plants with different treatment groups

H_1=There is statistical difference in fresh root weight of plants with different treatment groups

Result: P< 0.05, so result is statistically significant

Conclusion: T4=AI35(Itahari)treatment exhibited better root growth of plants than control and other treatment groups.

ANOVA

Fresh weight (Day 60)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	187.000	1	187.000	1.260	.024
Within Groups	593.506	4	148.377		
Total	780.507	5			

P=0.024

H_0= There is no statistical difference in fresh weight of plants with different treatment groups

H_1=There is statistical difference in fresh weight of plants with different treatment groups

Result: P< 0.05, so result is statistically significant

Conclusion: T2=AD2(Dharan) treatment exhibited better root growth of plants than control and other treatment groups.

ANOVA

Dry weight (Day 63)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29.562	1	29.562	5.915	.05
Within Groups	19.991	4	4.998		
Total	49.553	5			

P=0.05

H_0= There is no statistical difference in dry weight of plants with different treatment groups

H_1=There is statistical difference in dry weight of plants with different treatment groups

Result: P< 0.05, so result is statistically significant

Conclusion: T2=AD2(Dharan) treatment exhibited better root growth of plants than control and other treatment groups.