UTILIZATION OF ENDOPHYTIC BACTERIA AS A BIOLOGICAL CONTROL AGENT OF FUNGAL PATHOGENS AND ITS PLANT GROWTH PROMOTING ACTIVITIES IN *Solanum lycopersicum*

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This is to certify that the research work entitled **“UTILIZATION OF ENDOPHYTIC BACTERIA AS A BIOLOGICAL CONTROL AGENT OF FUNGAL PATHOGENS AND ITS PLANT GROWTH PROMOTING ACTIVITIES ON TOMATO”** has been carried out by **Ms. Manju Adhikari** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. I, hereby, recommend this thesis for final evaluation.

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This matter embodied in this report has not been submitted for award of any other degree.

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LIST OF ABBREVIATION

PGPR	Growth Promoting Rhizobacteria
PGPE	Plant Growth Promoting Endophyte
Mos	Microorganisms
PDA	Potato Dextrose Agar
NA	Nutrient Agar
LB	Luria Bertani
rpm	revolution per minute
Mins	Minutes
°C	Degree Celsius
LCMS	Liquid Chromatography Mass Spectrometry
Gms	Grams
L	Litre
ml	milli Litre
µl	micro Litre
CMC	Carboxy Methyl Cellulose
M	Molar
NaCl	Sodium Chloride
$NaNO_3$	Sodium Nitrate
K_2HPO_4	Di Potassium Hydrogen Phosphate
$MgSO_4$	Magnesium sulphate
KCl	Potassium chloride
$FeCl_3$	Ferric chloride
H_2SO_4	Sulphuric acid
$HgCl_2$	Mercuric chloride
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
%	Percentage
Taq	*Thermus aquaticus*

HCl	Hydrochloric acid
dNTP	deoxy ribonucleotide triphosphate
Kb	Kilobyte
V	Volt
ng	nano gram

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Non consumables and instruments

All the apparatus and instruments used during the whole experiment are:

Autoclave (Enaxy)

Autoclavable centrifuge tubes (Tarson)

Autoclavable Falcon tubes (Eppendorf)

Cuvette (Sigma)

Electronic Balance (Sartorius)

Electrophoretic Unit (Genet Bangalore)

Gel Doc (Alpha Imager)

Freezer [-80°C] (Blue Star, Thermo,electron Corp.)

Freezer [-20°C](Blue Star)

Refrigerator (LG)

Laminar Hood (Clean Air Atlantis Application)

Magnetic Stirrer (Remi)

Micropipette (Eppendorf)

Micro tips (Tarsons)

pH meter (Eutech Meter)

Spectrometer (Perkin Elmer)

Water Bath (Remi)

UV Transilluminator (Vilber Lourbat)

PCR Machine (Eppendorf)

Shaker (Kuhner, Switzerland)

Culture Tubes (Borosil)

Petri dish (Genaxy)

Conical flask (Borosil)

Glass Jars (Schott-Duran)

Incubator (Kuhner, Switzerland)

PCR tubes (Tarsons)

ABSTRACT

Bacterial endophytes are a class of microorganisms that are found in the inner part of plant, especially in the plant tissues without showing any detrimental effects on the plant growth and development. They are distributed widely and are found to be aiding plants growth and development by overcoming various biotic and abiotic stresses. The main aim of this study was to isolate endophytic bacteria as biological control agents against fungal pathogens and its utilization as plant growth promoters. For this, ten endophytic bacteria were isolated from the leaves of different tomato plants from JNU campus. Preliminary screening of all the isolates was carried out against tomato plant pathogens *Alternaria alternata*, ITCC 6134. The most promising strain was further subjected to molecular characterization by 16s rRNA analysis. The liquid Chromatography Mass Spectrometry technique showed the biological active compounds present in the bacterial exract of BBB3 which might have played role in inhibiting fungal growth. Different tests for enzymic activity like cellulase, phosphatase and heavy metal resistance (Mercury and Cadmium) were done. The *in vivo* plant growth promoting activities on tomato seedling and seeds were carried out in a greenhouse to observe the efficacy of isolated endophyte directly. The isolated bacteria coded BBB3 among ten showed the efficient fungal inhibition as it suppressed the pathogen invitro. BBB3 also showed the degradation of cellulose media, phosphate solubilization activity and resistance to a high concentration of Mercury and Cadmium. The greenhouse evaluations of the endophyte showed significant ($p \leq 0.05$) biological control efficacy, higher growth and yield components of tomato plant. Furthermore, the metabolomics profile of the active compound could be exploited for large scale production of natural and environmental friendly biological control agents for the replacement of synthetic chemical agents and directing towards sustainable agriculture.

Keywords:*Endophytes, Heavy metal resistance, sustainable, plant growth promoter*

CHAPTER 1. INTRODUCTION

1.1 Background

As the human population is increasing in a rapid fashion, demands are also increasing for food, shelter and related requirements. Agriculture is the only medium that will feed the growing population and to increase the crop production different strategies are applied like the use of organic and inorganic fertilizers. But the harsh use of these non-ecofriendly techniques are degrading the soil quality and resulting environmental pollution. So, to replace these chemical fertilizers interests are developed in using biotic measures which will solve the problem and do not result negative impacts on the concerned parameters (Hassan, 2017). Endophytes are the organisms which are found in the inner tissues of plant for a small part or for completion of their life cycle. They reside in the tissues of plant without any symptomatic behavior (Hassan, 2017).

These can be bacteria, fungi, actinomycetes and so on. They have the ability to colonize the inner tissues either by direct methods or indirectly. Endophytes enter into the plants through different routes, majorly through the roots. Also, they can enter through the aerial parts of plant like flowers, stem, and cotyledons. They also enter through germinating radicles, also through secondary roots, stomata. One way of entrance is also the damage of foliar parts. They are not found to be localized in one region of the plant that means, if they enter from one region, they can be found in the whole tissues of the plant or some can be tissue specific as well (Zinniel et al., 2002). They are found in almost every region of plant body like leaves, stems, barks, roots, flowers, fruits, seeds etc. They are found not to show any pathogenic effects to the host plants (Fouda et al., 2015).

The bacteria that are found inside the plant tissues and live either neutrally or symbiotically are known as symbiotic bacteria. These bacteria can be gram positive or negative. Bacterial species can vary from plant to plant or similar bacteria can be found in many plants. They can be isolated from different plant parts especially internal parts by surface sterilization techniques . Also fungi are found in endophytic form which generally belong to ascomycetes group; they are also aiding plants like endophytic bacteria. They are found in high number which inhabit around 300,000 species of plants. Both the endophytic organisms, bacteria and fungi produce different metabolites which has application for the growth and development of plants. These compounds can act as antimicrobial agents against the pathogens, which means, as bactericidal against bacterial pathogens and as fungicides against fungal pathogens (Fouda et al., 2015).

Another important concern of today's agricultural condition is crop loss due to infection by pathogens that may be bacterial or fungal. As for example, *Fusarium* is one of the fungal pathogens that cause disease on economically important plants and is a soil borne pathogen. Different strategies are applied to control such pathogens like the use of bactericidal and fungicidal agents but still the outcome is less. So the bacterial endophytes which are the normal flora of plant are chosen to solve the problem. They are taken as biocontrol agents which control the plant pathogens (Ohike et al., 2013).

Another important role of these endophytes is aiding in plant's growth and development. They are taken as Plant Growth Promoting Bacteria. They carry out different function like nitrogen fixation forming root nodules which help plants to take nitrogen from the soil. As nitrogen is an important nutrient for plant so these endophytes help plant to uptake nitrogen from soil which is generally present in unabsorbable form. Some help plant by stimulating plant growth hormones like auxins, cytokinins, gibberellins etc. Also iron is another important element for plant but the iron present in soil is not directly absorbed by plant so these bacteria help to uptake iron from soil by forming siderophores. For the control of plant pathogens they secrete antibiotics, toxins and also inhibit them by competing for nutrients and ecological niche (Botta et al., 2013).

Not only the rhizospheric region is rich with microorganisms, phyllospheric region is also found to harbor huge number of micro-organisms. Phyllospheric region contains bacteria as well as filamentous fungi. Generally the bacteria which are found on the surface of plants are known as epiphytes and oppositely the one which dwell the inner tissue region are endophytes. The population density of endophytic bacteria is found to be varying from 102 to 109 which depend on various factors like plant taken, the part of plant taken, condition of plant, stage of development of plant, the plant genotype, interaction of plant with environmental factors like soil type, climate and other (Oliveira et al., 2002).

The distribution of the beneficial endophytes help plant in nutrient uptake and exchange. They help plant for solubilization of inorganic phosphate that is not absorbed by plant into soluble form. Another use of these organisms can be a mission to reduce global threat which are resulting due to anthropogenic activities that is ,the accumulation of heavy metals and polycyclic aromatic compounds which is contaminating the environment and the ecosystem. All forms of life are highly affected by this. These are resulting contamination of every component of environment from soil, air to water which is ultimately hampering health of human and animals. Majorly the heavy metal contamination are because of Cadmium which are result of industrial practices and human activities like the use of chemical fertilizers, burning of fossil fuels etc. So to control this global threat steps should be taken and for this, these bacterial endophytes are showing a great role. Plant degrade this harmful chemicals into less harmful components with the help of microorganisms by the process of phytoremediation ((Jeelani et al., 2017).

Tomato is a major vegetable crop that has achieved tremendous popularity over the last century. It is grown in practically in every country of the world - in outdoor fields, greenhouses and net houses. The tomato plant is very versatile and the crop can be divided into two categories; fresh market tomatoes and processing tomatoes. In both cases, world production and consumption has grown quite rapidly over the past 25 years.

Tomatoes, aside from being tasty, are very healthy as they are a good source of vitamins A and C, they also contain Lycopene; which is a very powerful antioxidant that can help prevent the development of many forms of cancer. Cooked tomatoes and tomato products are the best source of lycopene since the lycopene is released from the tomato when cooked. A raw tomato has about 20% of the lycopene content found in cooked tomatoes. However, raw or cooked tomatoes are considered the best source for this antioxidant (http://www.agrisupportonline.com/) (Guil-Guerrero and Rebolloso-Fuentes, 2009). Another advantage of this plant is that it is taken as an excellent model plant for study .The different phenomenon like disease condition, longevity of infection , cause of infection, effect of different environmental conditions such as stress conditions of heat, cold and so on. Their growth pattern is also another interesting subject to study (role of growth hormones in their growth and development. They produce different metabolites and the study of the function of those metabolites is also one major subject of keen interest (Gouda et al., 2016). Also it is one of the most popular vegetables worldwide which is used in either cooked form or simply as raw (salad). But there are many limiting factors for its cultivation out of which infection is one of the major cause. This will decrease the yield and ultimately hamper the overall productivity. The pathogens responsible for this are bacteria, fungi, nematodes, virus, and insects and so on. Again, (2002) presented major diseases of tomato caused by 24 fungi, 7 bacteria, 10 viruses, 3 viroid, and multiple nematodes. In Japan, 41 fungi, 10 bacteria, 1 phytoplasma, 15 viruses, and 14 nematodes have been reported to be pathogens of tomato, most of which are distributed worldwide (The Phytopathological Society of Japan 2000).

a b

Figure 1.1: Result of fungal pathogen infection on tomato a: Early blight b: Late blight

1.2 Current studies

In the recent years study of endophytes have been carried out a lot and the study majorly focusses on their role in combating plant pathogens and improving growth activities but the key point behind this activity of endophytes is still missing so the researches are going on. Their role as biological control agents (mechanisms) against pathogens of various strains is under study (Pavithra et al., 2012). Furthermore studies are carried out on their applications in industry, agriculture, medicine (Ryan et al., 2008). Similarly transmission and colonization of endophytes in their host plants are another aspect of study (Frank et al., 2017).

1.3 Research Hypothesis

a) Null hypothesis

$H_0$1: Bacterial endophytes isolated from one host play an important role in same host defense mechanisms .

$H_0$2: Bacterial endophytes have direct role in Plant Growth promoting activities.

b) Alternative hypothesis

$H_1$1: Bacterial endophytes isolated from one host have no role in same host defense mechanisms.

$H_1$2: Bacterial endophytes have no direct role in Plant Growth Promoting activities

1.4 Objectives

1.4.1 General Objectives

I. Isolation and screening of bacterial endophytes against fungal pathogens (*Alternaria alternata*) of tomato plant.

1.4.2 Specific Objectives

I. Investigation of biological control activity of bacterial endophytes against fungal pathogens on agar plates

II. Molecular characterization of the isolated bacteria using 16s rRNA sequencing method

III. Submerged fermentation, separation and extraction of metabolites

IV. Evaluation of enzyme activity of the isolated bacteria

V. Evaluation of plant growth promoting activities of bacterial endophytes.

1.5 RATIONALE OF STUDY

The role of endophytes as plant growth promoting agents is a highly discussed subject matter but there are other various functions which need to be studied and explored. Combined role of bacterial endophytes as biological control agents and plant growth promoting agents using tomato plant is the major case of study. Biological control activity of bacterial endophytes is already analyzed by many researchers but using fungal pathogens of tomato plant is not done yet. Also the isolation of endophytes is done using different samples and the test is done using different other plant species but here tomato leaf is taken for the isolation of endophytes and other plant growth promoting activities is also analyzed using tomato plants only. The biological control activity of endophytes isolated from tomato was checked against fungal pathogens of tomato namely*, Alternaria alternata, Sclerotium rolfsii, Fusarium oxysporum* which is also not done before. To understand the mechanism of defense against pathogens, analysis of the bacterial extract is also done using chromatographic and spectrometric technique (LCMS). One of the major focus of our study was to understand the other role of these endophytes in controlling environmental pollution against heavy metals and experiment was set up and the test was done against high concentration of Mercury and Cadmium. The ultimate goal of this research is to isolate the endophytes with many advantages which can replace the harnessing chemicals used by farmers to enhance agriculture.

CHAPTER 2. REVIEW OF LITERATURE

The very minute living organisms (microorganisms) have played a major role in the discovery and finding of new things for the welfare of human and animals like for discovery of new drug, industrial and other agricultural applications. Only a fraction of microorganisms is identified till now, that is, one percent of bacteria and five percent of fungi and rest are still to be identified. But this small quantity is aiding for human welfare majorly (Qadri et al., 2013).

2.1 Plant growth promoting bacteria (PGPB)

The interaction between plant and microorganisms have always been a key point of study. This type of interaction can be both positive and negative where the positive interaction results in plant growth whereas negative results in the opposite effect with respect to bacteria. PGPB are bacteria which promotes the growth and development of plant along with protection via different mechanisms (de Souza et al., 2015).

PGPB forms

There are especially two types of PGPB namely extracellular and intracellular. The bacteria inhabiting the outer surface are simply the extracellular and the later resides inside the plant body. There are different types of interaction between plant root and soil bacteria like endophytic, symbiotic, associative and free (de Souza et al., 2015). Endophytic PGPB are good inoculant candidates, because they colonize roots and create a favorable environment for development and function. Non-symbiotic endophytic relationships occur within the intercellular spaces of plant tissues, which contain high levels of carbohydrates, amino acids, and inorganic nutrients (Bacon and Hinton, 2002). By name only they are growth promoters of plants and they do so by involving themselves in various processes. They not only promote but also protect the plants (Glick & Glick, 2012).

PGPB undergoes various interaction with host for survival like rhizospheric (binds to root or seed surface) , phyllospheric (binds to leaf or stem surface) , endophytic (resides inside the tissues of plants) and symbiotic (found in root nodules) (Glick, 2014).

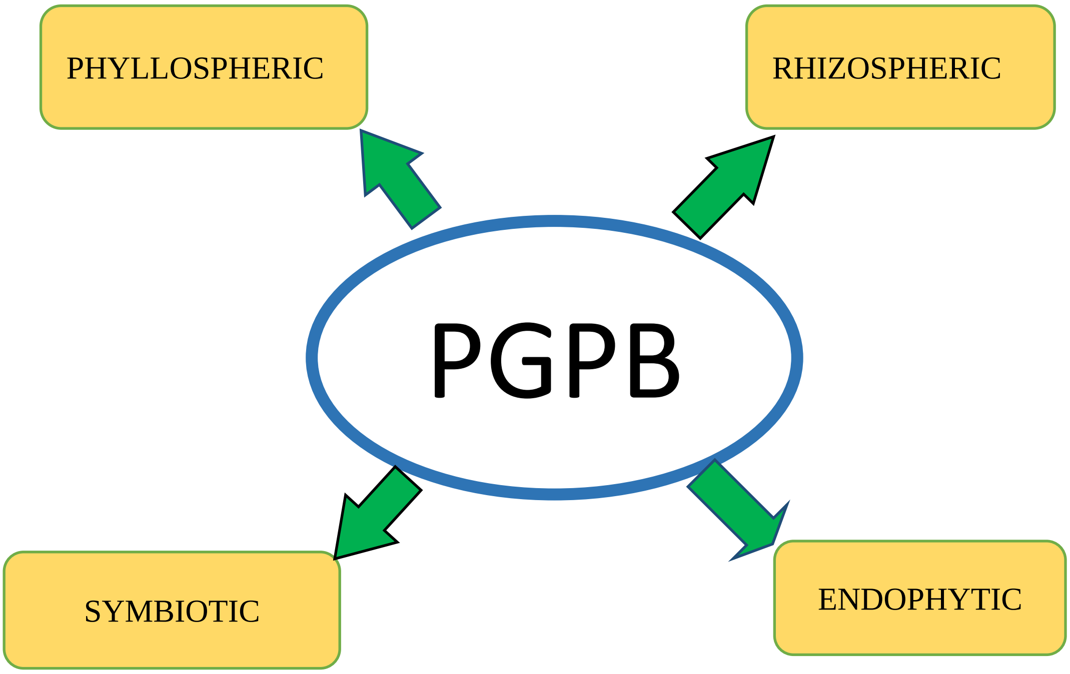


Figure2.1: Different types of interaction followed by PGPB

2.2 Plant growth promoting rhizobacteria

Rhizosphere is the region of the soil near the root where different kind of microbial life are found among which some are beneficial which promote the growth of plants known as plant growth promoting rhizobacteria. The number of bacteria is found to be high in the rhizospheric region because of the abundancy of nutrients required for them like sugars, amino acids, organic acids and other plant root secretions which might aid for their colonization (Chaparro et al., 2013). They are simply the soil dwellers which aid in plant's growth via different mechanisms like nutrient uptake, mineral solubilization like Phosphorus, Iron, Magnesium, protecting from any kind of pathogens (Ahemad & Kibret, 2014). These rhizospheric region colonizers help plants directly by involving in plant stress control, rhizoremediation, phytohormones secretion which ultimately help in plant promotion and indirectly as biological control agents by developing resistance against pathogens (induced systemic resistance, systemic acquired resistance), competition for food, space, secreting antibiotics, and so on. Some of the common PGPR includes: *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Rhizobium* and *Serratia*. These are the main subjects of research in this present era (Dinesh et al., 2015).

2.3 Endophytes

Endophytes are either bacteria or fungi that are found in inner tissues of plant and do not show any unfavorable effect for plant growth and also can be isolated through disinfection techniques. They are found inside the plant tissue either intercellularly or intracellularly without hampering the plant (Miliute et al., 2015).]. Endophytes carry importance because of their role in plant growth and defense mechanisms. Endophytic mode of microbial life and their ecological function has been a point of attraction for research and study in recent years. The endophytic microbial diversity of beneficial organisms may be more as there are nearly 300,000 terrestrial plant species, whereas each plant can host more than one type of endophyte. They can be bacteria or fungi ,their occurrence is found almost in every part of the plant like root, leaf. Usually the association between plant and these mos. can be obligate or facultative and symbiotic as well where the two groups benefit each other mutually (Nair & Padmavathy, 2014). Also some forms are found to be pathogenic as observed by (Shekhawat et al., 1984) in case of potato. The non-pathogenic bacteria turns into pathogenic if there will be hinderance from external factors like environment fluctuation (Sturz et al., 2000).

It is that found that both pathogenic (Slippers & Wingfield, 2007) and nonpathogenic endophytic bacteria co- existing within potatoes, and speculated that the populations of pathogenic bacteria within plant tissues remained quiescent until an external event triggered disease development. Such latent infections may be a function of low pathogen number (Rosenblueth & Martínez-romero, 2006) or, alternatively, certain bacterial species may become pathogenic under certain conditions.

De Bary introduced the term endophyte in 1886 for microorganisms (fungi, yeast, and bacteria) that colonize the tissues of plant (De Bary 1884).

Occurrence of endophytes

The presence of endophytes is ubiquitous as there are around 300,000 plant species on Earth, the diversity of these mos. increases. They are found in different types of plants like monocots, dicots, conifers and so on also in different parts like roots, stems, flowers, buds, vegetables and so on. Even a single plant can harbor various types of microflora and these mos. can have a wide host range. There are various factors that play role in diversity of the endophytes distribution like microenvironment of soil, plant variation of different geographical distribution (Stępniewska & Kuåniar, 2013).

Distribution of endophytes

Generally endophytes are distributed into three types based on their interaction with host. They are as follows:

- Obligate endophytes: These are completely dependent on the host (plant) for their survival and growth and cannot develop outside of plant and their transmission occurs through seed only.
- Facultative endophytes: These are the opportunity seekers kind of endophytes that means they are found freely living outside the plant also and if find any chance to penetrate the plant they proliferate inside also. They can enter via cracks or wound and sometimes through mutually coordinated infection. They are found to be having infection machinery for their colonization.
- Passive endophytes: These are not plant dependent for their growth and proliferation and do not need to colonize the plant tissues. These are found to provide less aid for plant growth because they do not possess any mechanism to enter into plant and if enter also, they do passively via wound. This mechanism is found to be less competitive for colonization because no cellular machinery is required for the host colonization. (Est & Unfield, 2013).

2.4 Bacterial endophytes

These are the bacteria found inside the tissues of plant which do not have any detrimental effects on plant growth and development, they are taken as an important discovery for the agriculture improvement and development. These small life have given tremendous benefits to the host either by simple ways or via complex mechanism from nutrition, defense to production of different metabolites (Bhore et al., 2010). The another keen aspect of study is the symptomless nature of endophyte's colonization in the plant tissues. Also these bacteria are postulated to be the intermediatory organisms between saprophytes and pathogens of plant (Hallmann et al., 1997).

Occurrence

Bacterial endophytes are in direct contact with plant cells as they live inside plant tissues which therefore results beneficial role as compared to rhizospheric bacteria even though the bacteria residing in the rhizosphere also can enter and colonize the plant roots. As rhizosphere is the common residing location for all kind of bacteria so there is the competition for ecological niche, nutrients and this competition results in the colonization if plant tissues in either beneficial or pathogenic way which have positive or negative effects on plant growth and development. In other sense bacterial endophytes are another form of rhizospheric bacteria or just subset in simple terminology (Chaturvedi & Singh, 2016). Endophytes are also found in fruits and flowers but the occurrence is found to be very less which confirms that endophytes are not only the root region dwellers but also the phyllospheric ones (Compant et al., 2011).

It is surprising fact that how these bacteria get colonized inside the plant and there are different mechanisms suggested for the entry of bacteria inside plant tissues among which the entry through roots is common .They enter via vertical cracks and wounds through vertical and lateral root hair cells, also rhizobia are found to enter forming root nodules whereas some bacteria penetrate through stomata ,young stem ,germinating radicles (Santoyo et al., 2016).

Bacteria also can hydrolyze the wall of plants producing hydrolytic enzymes like cellulase, lipase, hemi-cellulase, pectinase and find out way for penetration (Hallmann et al., 1997). Endophytic fungi are the higher group of organisms which are found inside the plant tissues. Majorly the anamorphic fungi and ascomycetes group are found to be living as endophytic life. Similar to bacteria they also do not cause any notable damage to the plant. They are also assisting plant by producing different bioactive compounds.

So as a key point of study the cellulose degrading enzyme cellulase are taken. This enzyme converts the complex lingo-cellulosic biomass into fermentable sugars. Cellulase consists of three enzymes:

- Endoglucanases: which hydrolyze the amorphous region of the cellulose,
- Exoglucanases: which release the cellobiose units from non-reducing ends and
- B-glucosidases: which cleave the short chain of cello-oligosaccharide into simpler glucose units.

Some major organisms which are used for the this enzyme production are *Trichoderma, Penicillium, Aspergillus* which come under fungi and bacteria such as *Clostridium, Bacillus, Streptomyces* are getting major emphasis (Liang et al., 2014).

This cellulose degradation is one of the way to control the organic contaminants and conversion of them into some useful forms like biofuel, ethanol. Microorganisms take this contaminants as their substrate or food. Also the enzymes produced by them are having huge application in industrial sectors (Kaur & Arora, 2012).

When plants develop wound they secrete certain metabolites which attract bacteria and in response to that they enter .There are other route also like stomata ,lenticles, radicles etc., this is usually seen in young leaves and stems (Santoyo et al., 2016). Most of the studies have shown that the major endophytic organisms belong to the rhizospheric ones rather than other one and once they get entry from root parts mainly from root hairs either via natural opening or through injuries. Later they transfer to different plant parts .Most of them are found to be closely related with saprophytes (Scott, 2016).

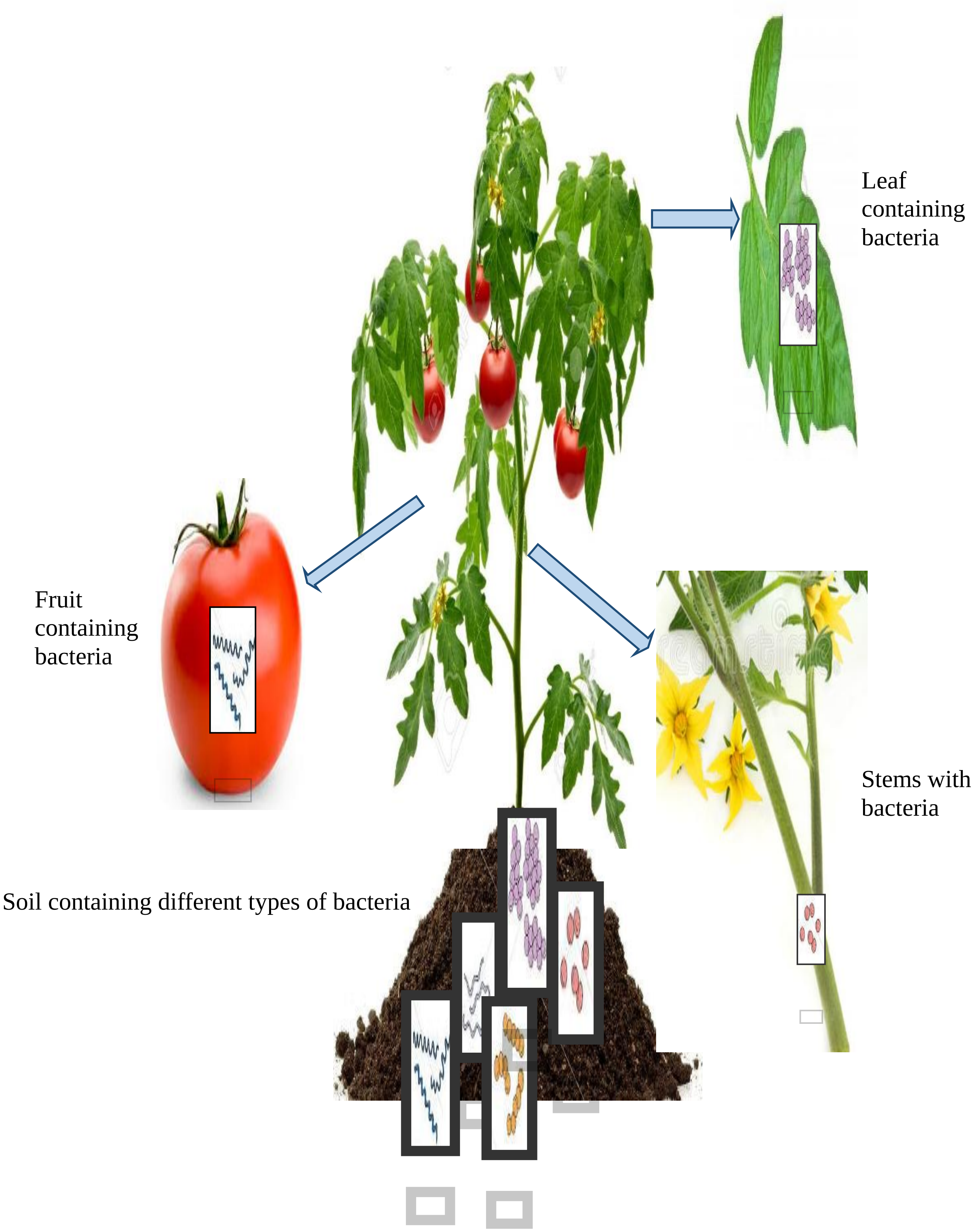


Figure 2.2: Mode of entrance of bacteria into plants through roots

2.5 Niche

In the rhizosphere region of the soil the huge number of microorganisms are found and also the interaction between them is found to be more when compared to the other region of the soil. There is found to be the interactions between microorganisms and plant and also in between themselves. These interactions are aided either by plant secretion like root exudates or microorganisms. The plant secretes many compounds which not only attract the microorganisms but also help for their growth. Usually several factors affect the colonization and alteration in any factor can convert the interaction type also, such as, non-pathogenic bacteria can turn into pathogenic ones. Generally survival of either components is highly determined by the soil factor that means the type of soil. It can be acidic, basic, supply of nutrients and so on. The plant factors also determines the microbial colonization and survival. The capacity of microorganisms to make it an endophytes also determines the interaction type with the host (Gaiero et al., 2013).

2.6 PGPE isolation

Isolation of endophytes is a complex process which depends on the type of organisms we want to isolate. As the plant body consists of different parts like leaf, stem, root, flower and so on and the types of microorganisms vary along with that. The mos. that are found in one part of the plant may or may not be found in another part. So, it is very necessary to distinguish what parts are suitable for the isolation of desirable mos. And also the endophytes are found in inner part of plant cell, it should also be taken under consideration. However there are different methods applied for the isolation, among which the use of Sodium hypochlorite is very popular. Sodium hypochlorite is a disinfectant which is used for the surface sterilization of the plant parts and the tissues are mostly rinsed with Sodium thiosulfate which also removes the excess sodium hypochlorite (Hassan, 2017).

Bacterial endophytes are isolated from various types of plant that may be woody such as oak, either monocots or dicots, herbs, edible or inedible plants, economically important plant or medicinally important plants by applying the hypochlorite disinfection techniques.

2.7 Applications

Plants are sessile organisms that means they are unable to move from one place to another. They even cannot move in response to any danger condition like stress that may be biotic or abiotic stresses. However, the nature have provided indirect solution to this problem where a beneficial interaction between host plant and some useful microorganisms occurs. This mutualistic network make plant able to withstand the stress conditions by producing special compound for defense, communication compound, allelochemicals and so on (Eckelmann et al., 2018).

2.7.1 As Plant growth-promoting endophytes

Bio fertilization

Plants need nutrients available in the soil for their growth and some bacteria can help them in uptake of those nutrients. These bacteria play a major role in increasing the fertility of soil so are known as bio fertilizers. They perform this function by different mechanisms like biological nitrogen fixation. As nitrogen is one of the essential nutrient but is less available to plants. This should be converted into absorbable forms like nitrate and nitrite .The bacteria develop a mutual relationship with plants (host) and convert atmospheric nitrogen into absorbable forms. Generally this kind of endosymbiosis is observed in leguminous plants which fix nitrogen by forming root nodules. Inoculation of non-leguminous plants with this bacteria enhances nitrogen fixation and hence increase fertility of soil (Murray, 2011). Also these bacteria help in the uptake of other essential nutrients for plants like Ca, K, Fe, Cu, Mn, Zn. They increase the root surface area for absorption, maintains the PH of the soil. The productivity of highly consumed food like wheat, sugarcane, paddy, maize which do not form any kind of association can be increased by co-inoculation with this growth promoting bacteria (Pérez-Montaño et al., 2014).

These plant growth promoting bacteria which perform different functions as compared to biocontrol agents, these bacteria do not control the plant pathogens but helps directly by helping plants in nutrient solubilization and uptake (Ryan et al., 2008). Also these help plants by osmotic adjustment, root morphology modification. They also act as supplier of useful vitamins.

2.7.2 Natural products from endophytic bacteria

Most of the endophytes are taken under consideration because of their capacity of producing various kind of secondary metabolites which includes antibiotics ,antimicrobial compounds, antiviral compounds, anti-cancer agents and so on (Ryan et al., 2008).

Cellulase activity

One of the plant biomass which is found abundantly on the earth surface is cellulose. It is also taken as the main component of waste material from domestic to industrial based on agriculture. The major waste of plant including cellulose are the plant parts like stem, stalk etc. These waste are taken as a renewable source for the production of essential compounds. The major research are going on the production of the enzyme which can degrade this cellulose (Hussain et al., 2017). Cellulose is the polymer chain of glucose units which are having b-1,4 glyosidic linkages. Presently variety of microbial species are screened for the degradation of this complex polymer (Johnsen & Krause, 2014).

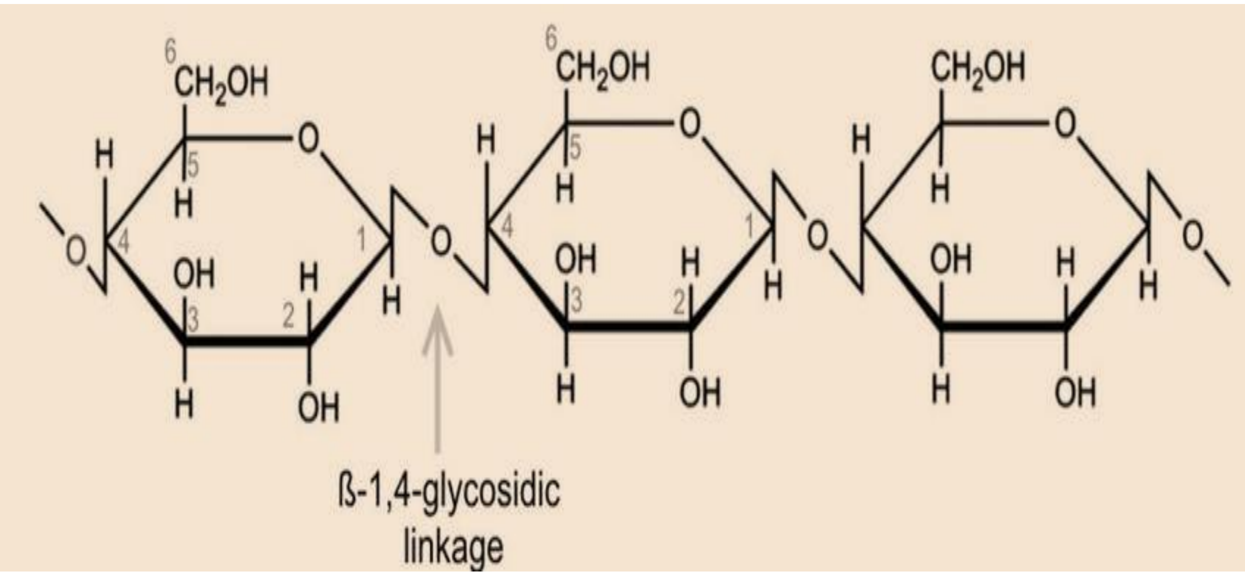


Figure 2.3: Structure of cellulose

Phytoremediation

The increasing population is resulting increasing human activities like industrialization, more and more agronomic activities and others which is leading to global problem of pollution and affecting health of animals, human and also harming plants and other existing creatures. Among the various pollution metal (toxic) acquisition is one. So several techniques are applied to minimize this and among the all Phytoremediation carry a high weightage which is safe and easy technique which use plants for the remedy and the effectiveness of plants can be increased by using plant assisting bacteria (Ullah et al., 2015). The use of heavy metals like mercury, cadmium, nickel has led to deposition which ultimately degrade the environment and also the plants. The endophytes are found to be having a beautiful property of phytoremediation (Ryan et al., 2008). Some can withstand the high concentration of such metals and protect plant from the toxic effects of them because plants inoculated with such bacteria, develop a kind of tolerance in them also. Some can degrade the heavy metals also which are supposed to have such metal ions degrading genes (Siciliano & Germida, 1999). Some bacteria due to their genetic capability, they make themselves grow in polluted soil that might have resulted from human activities. They carry the capacity of tolerating the pollutant's concentration if incase high also (Ullah et al., 2015). The role of endophytes in phytoremediation is highly concerned topic and the steps are taken for the solution of polluted soil especially with heavy metal pollution but the information is however not up to the mark (Ma et al., 2015). As a group organic compounds fall under the pollutant of the environment and ecosystem, polycyclic aromatic compounds are one of that pollutants of either terrestrial or aquatic ecosystems. This usually results mutation, cancer and other so many genetic deformities in affected organisms. Many technologies are developed to solve this global problem however using plant and microorganisms which have the ability to degrade these pollutants into non harmful compounds are under consideration (Sivaram et al., 2018).

2.7.3 Modulating the Effects of Environmental Stress

Growth of plant is determined by various parameters and some unfavorable conditions halts the growth of plant (Glick & Glick, 2012). These conditions can be simply termed as stress conditions. Stress can be biotic (living parameters) and abiotic (nonliving parameters). Biotic stress includes pathogens of plant like bacteria, fungi, and virus whereas abiotic stress includes unfavorable temperature, salinity, drought, flood, light, heavy metals, radiation etc. (Glick et al., 2007).

Phytostimulation

Phytostimulators are the compounds which aid plants for their growth and these can be synthesized by PGPE. Majorly the growth hormones like indole-3-acetic acid (IAA), gibberellins (GAs), cytokinins and certain volatile organic compounds are produced by these bacteria (Pure et al., 2016). IAA helps in root formation and elongation which help plants for development of roots (Selim, 2016) shoot, decreasing senescence. These phytohormones play role in colonization and signaling also (Khan and Doty, 2011); (Mercado-blanco & Lugtenberg, 2014).

Antagonism

Antagonism is the phenomenon of avoiding the growth of other organisms which is carried out by different steps like producing antibiotics, toxins and other inhibiting substances. Also, they produce the hydrolyzing enzymes against the pathogens such as cell wall degrading enzymes (chitinase, glucanase) (Pandey et al., 2017).

Systemic response induction

These are the systemic responses developed by the plant in response to any pathogen. There are two types of responses: 1) Induced Systemic Response (ISR) and 2) Systemic Acquired Resistance (SAR). ISR is response developed when the useful bacteria colonize with plant and result resistance to pathogens (Tam et al., 2018). This ISR response is developed by nonpathogenic bacteria against pathogens which is dependent on signaling in plant, that is, jasmonic acid and ethylene signaling (Loon et al., 1998). SAR response is developed after the infection by pathogens and salicylic acid is the main signaling molecule. For defense, elicitors play role and some of the known elicitors are salicylic acid, cyclic lipopeptides, antibiotics, signaling molecules like N acetyl homo serine lactones etc. (Kloepper & Ryu, 2006); (Berg 2009).

Competition for iron

As iron is important element for all forms of life and iron found in ferric forms are unavailable to microorganisms. In such cases some bacteria can convert ferric ion into ferrous form that is absorbable by forming siderophores. The capacity of producing extracellular siderophore is found in some PGPR which cause iron deprivacy in pathogens which cease the growth of pathogens and increases the crop productivity (Lacava et al., 2013); (Aswathy et al., 2014).

Biological Control Agents

The biological agents especially organisms that are used to control the growth of unwanted organisms is known as biological control agents. These control agents can be bacteria, fungi, nematodes and so on. These are the natural controllers of pathogens for their survival .They suppress or kill their pathogens via predation, competition for niche, nutrients and so on (Bale et al., 2008).

Importance of biological control agents

Due to increase in population and the increase in food demand has provided evidence that many parts of the world are reaching their productive maximum for agricultural land use (OECD-FAO, 2012). To overcome this global problem some measures are taken like the haphazard use of chemical fertilizers, pesticides which has hampered the environment by stressing the ecosystem, global warming, all sorts of pollution (land, water, air) which endangers future yields (OECD-FAO, 2012). The use of toxic metals and organic compound are also one of the major causes for the environment pollution .These are also resulting the resistant pathogens against any treatment so there should be an environment friendly technique to overcome this problem without deteriorating other parameters for sustainable agriculture like use of biological control agents against pathogen (Glick, 2010). Biological control is taken as an alternative or a supplemental way of replacing the use of chemicals in agriculture and increase yield in eco-friendly manner(Compant et al., 2011).

Table2.1 :Showing the benefits of Biological control Agents(Sharma et al., 2013).

Biological control agents					
Affordable as compared to other methods	**Easy to handle**	**Longevity in application to the crops**	**Non toxic to plants**	**Environmental friendly (do not affect health of human and animals)**	**Effective and promising agents**

2.8 Plant pathogens

As the world population is directly dependent on agriculture but the present agricultural condition is not sufficient to feed the growing population and one of the important reasons for this is plant degrading pathogens. These pathogens directly harm the plant and decrease the crop yield and the intense use of chemical pesticides for the control of these pathogens is increasing the problem more and more. The pathogens are becoming resistant to pathogens. There are different types of plant pathogens such as:

2.8.1 Bacterial pathogens

These plant degrading bacterial pathogens are one of the most reason for crop loss in biotic agent forms. Different strategies are applied to control these pathogens but are always insignificant because due to lack of effective control agents. Also mostly the bacterial diseases spread through contaminated seeds and as seeds are used for the germination of plants many countries are facing the problem due to the use of contaminated seeds. The ability of these pathogens to colonize the seeds is one of the threats. So, there should be the development of new strategies to control this global problem (Makarovsky et al., 2018).

2.8.2 Fungal pathogens

Fungi are the higher group of organisms as compared to bacteria and are highly distributed in the ecosystem. They are found in various parts from soil, water, air and so on. They are an important part of nature and also influences the life of human, plant and animals. Some fungi are useful while some are found to be having negative effects on life of plants. Pathogenic fungi are one of the highest constituents for crop loss. They cause crop spoilage either before harvest or after harvest (Abd-Elsalam et al., 2010).

The main characteristic feature of these organisms are their different modes of reproduction, that is, sexually or asexually and the presence of different structures like mycelia, hyphae, These structures are somehow aiding the fungi to cause negative impacts on plant growth and development. The studies are going on how these structures are playing role in pathogenesis as detail knowledge is still lacking. Different techniques are applied for their identification like molecular technique, immunological, pathogenicity detection and so on, and also the new advances are studied in these techniques which will help for detail identification. Also Isozyme analysis, vegetative compatibility group (VCG) analysis and electrophoretic mobility of cell wall proteins have been shown to be useful for the detection of strains of some fungal pathogens Isozyme analysis, vegetative compatibility group (VCG) analysis and electrophoretic mobility of cell wall proteins have been shown to be useful for the detection of strains of some fungal pathogens (Narayanasamy, 2011).

Fusarium oxysporum is one of the plant pathogenic fungi which cause devastating loss of plants and crops. This is a soil borne ascomycete which especially invade the root of plants. More than a hundred plant species are found to be victimized by this pathogen. The general symptoms of infection are color change from green to yellow, wilting also sometimes cause death due to colonization of xylem vessels. This pathogen is contributing high for the crop loss in a significant manner. Various strategies have been applied for the control of this pathogen such as crop rotation, applying fungicides, breeding of resistant crops and also via application of biological control. But the attempts are found to be incomplete in controlling because the pathogen is undergoing various changes through environment or genetic which is making it resistant to any treatment and pollution is one of the major reason behind it.

The broad host range is also making it survive in persistent manner because it can easily accommodate its living within huge range of host (Wan et al., 2017). The disease caused by *Fusarium oxysporum* known as Fusarium wilt is found to invade the outer epidermis of the plant root and penetrates itself into the vascular tissue where it gets colonized especially in the xylem vessels. This ultimately results in water loss and cause stress to the plant. Some cause rot of root and destroy the whole plant while some strains of *Fusarium* produce brown coloration mainly by F. *oxysporum* f.sp. radicis lycopersici (Forl) (Abdesselem et al., 2016).

CHAPTER 3. MATERIALS AND METHODOLOGIES

3.1 Laboratory Setting

The works regarding my thesis was conducted in the laboratory of School of Life Science (lab no: 308) at Jawaharlal Nehru University, New Delhi, India.

Experimental plant: *Solanum lycopersicum* (Tomato)

The fungal pathogens namely *Alternaria alternata, Fusarium oxysporum, Sclerotium rolfsii* were provided by the laboratory itself.

3.2 Isolation and screening of bacterial endophytes

The bacteria were isolated from the leaf of tomato plant on agar plates that were collected from the field of SLS, JNU by following the protocol given by (Ohike et al., 2013) with slight modification. The leaves collected were healthy and free from contamination. The leaves collected were then thoroughly washed with running water and washed with sterilized water inside the laminar hood. After that 70% ethanol was used for disinfection process 30 secs. Then the leaf was treated with 1 % sodium hypochlorite solution 5 mins. Then the leaf was washed with sterilized water for five times. The leaf was then cut into pieces of size 5 mm with sterilized blade and placed on plate containing nutrient agar. The plate was incubated at 28°C for 24 hours. After 24 hours of incubation the colonies developed were subcultured into fresh NA plates.

For preliminary screening, PDA plates were used in which the bacteria were streaked on the plates against the fungi *Alternaria alternata*. It was wrapped with parafilm tape and incubated at 28°c for 24-48 hours. The plates were observed daily to check the growth. The inhibitory zone was measured and the one showing the best inhibition was screened for further analysis.

3.3 Molecular characterization of the bacteria using 16s rRNA sequencing method

a) Isolation of genomic DNA

The endophytic bacteria with the highest biological control activity BBB3 was selected and for molecular characterization, 16s ribosomal RNA (rRNA) sequencing method was used. The AxyPrep Multisource Genomic DNA Miniprep Kit was used to isolate DNA as described by the manufacturer’s manual. Electrophoresis of DNA thus extracted was done in 0.8 %

agarose gel and observed under UV- Transilluminator. For the comparison of DNA ladder of 1 kb size was used.

b) PCR amplification and sequencing of 16S rRNA gene from BBB3 isolate

For the amplification of the gene, PCR (Polymerase Chain Reaction) was done in a Thermal Cycler. The reaction mixture used consisted of 20 ng of genomic DNA, 2.5 U/50 µl of Taq DNA polymerase, and 5 µl of 10× Taq buffer (100 mM Tris-HCl, 500 mM KCl at pH 8.3), and additionally 200 µM dNTP, 10 pmol each of universal primers(forward primer 27F 5’AGAGTTTGATCCTG GCTCAG3’ and reverse primer 1492R5’TACGGTTACCTTGTTACGACTT3’), and 2.0 mM $MgCl_2$. For the amplification process, denaturation of the extracted genomic DNA was followed by the annealing of primers at 50°C for 30 s and extension at 72°C for 1 and 15 min. The amplified product of five microliters (5 µl) was analyzed by submarine agarose gel (1.2%) electrophoresis with ethidium bromide at 130 V for 30 mins followed by visualization under a Gel Doc/UV transilluminator. This was later gel purified using the Qiagen gel extraction kit and there-after sequenced with 100 ng/µl of 16S rRNA.

3.4 Biological control activity of the isolated bacteria against fungal pathogens

Dual culture assay method was used in which the bacteria and fungi were placed in the two opposite edges of the PDA plates (bacteria was streaked as line and fungi was taken with a sterile tip with the help of sterile tooth pick. It was incubated under same condition as before and the inhibitory distance was measured.

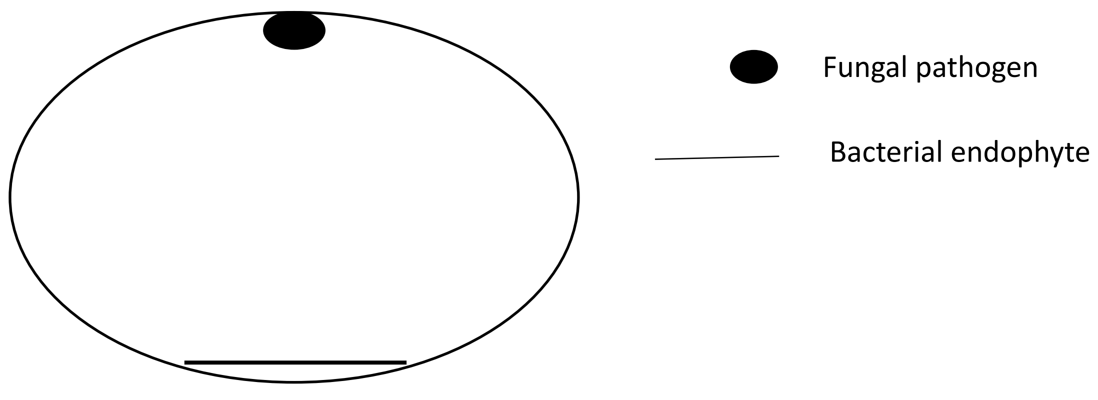


Figure3.4: Demonstration of dual culture assay using bacteria and fungi

3.5 Submerged fermentation, separation and extraction for Liquid Chromatography Mass Spectrometry (LCMS)

Luria Bertani Broth media was used which was prepared by dissolving 12.5 Gms of LB broth powder in 500 ml distilled and autoclaved at above mentioned conditions in section 3.2.

For inoculum development, firstly from the bacterial plates, one loopful organisms was inoculated in sterilized falcon tube containing 15 ml LB broth inside laminar hood and incubated in shaking incubator for 48 hours at 28°c, 220 rpm. Again, another LB Broth was used for inoculum preparation which was prepared by dissolving 5 Gms of LB media in 200 ml in conical flask and was autoclaved and after the media was cooled the 48 hours incubated culture of falcon tube is poured into the conical flask aseptically.

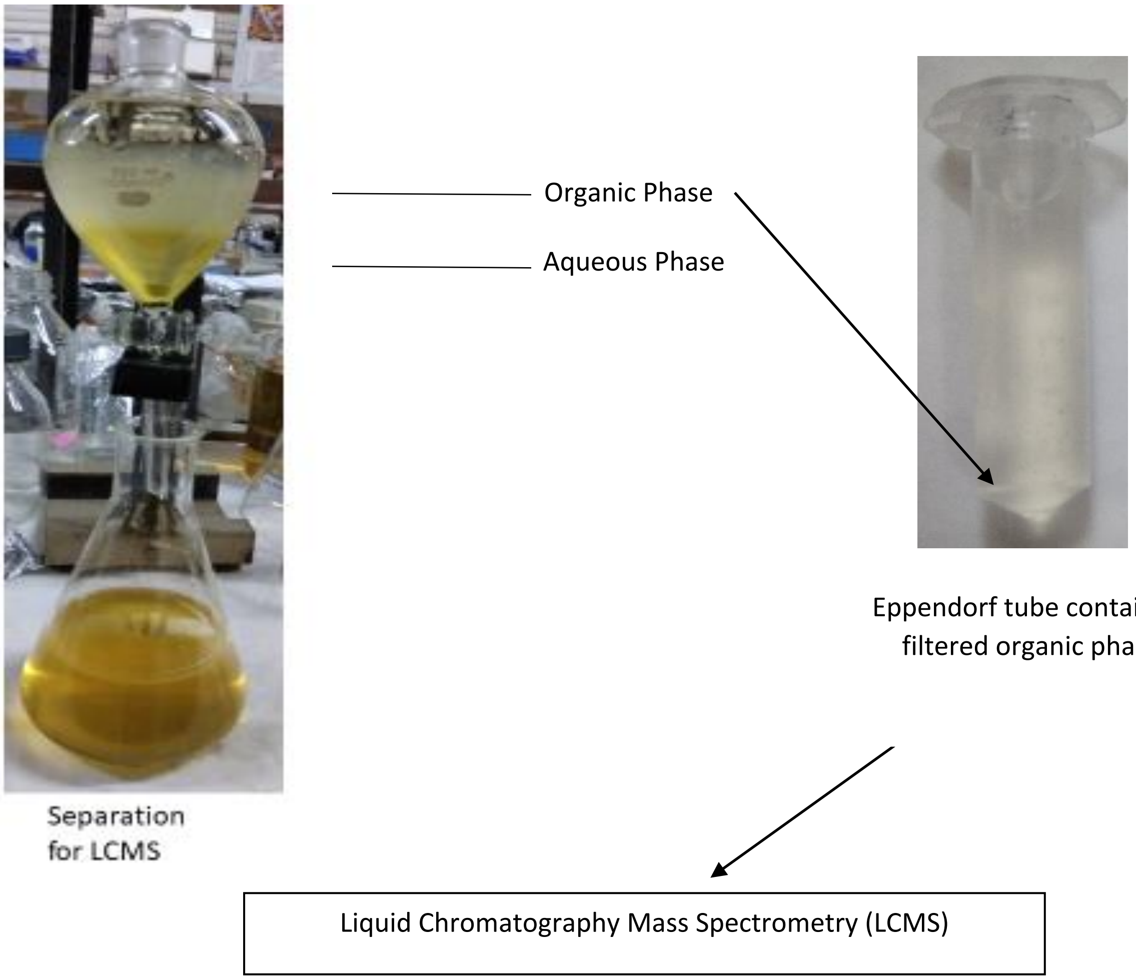


Figure 3.5: Separating funnel used for the separation of organic and aquous phase of culture.

(Aqueous phase discarded while organic phase was further filtered using a syringe filter and subjected for LCMS.)

This was then subjected to incubator shaker for 72 hours at 28°c, 180 rpm. After 72 hours the culture was ultra-centrifuged at 8000 rpm, 4°c for 10 mins using 250 ml GSA bottle and the supernatant was transferred to a clean conical flask while the pellet was discarded. Then to the clear solution which was 150 ml, equal volume of ethyl acetate was added and kept in magnetic stirrer for half an hour and then separation was done using a separating funnel in which the organic phase was taken and aqueous phase was discarded. To the separated solution sodium sulphate was added to remove the water and other impurities and the clear solution was taken in conical flask and 2 ml was drawn into an Eppendorf tube using a syringe filter. The clear solution was subjected for LCMS at Advanced Instrumentation Research Facility (AIRF, JNU, New Delhi).

3.6 Enzyme activity

3.6.1. Phosphate solubilization

Pikovskaya's agar medium is used for isolation of phosphate-dissolving strains (Pikovskaya, 1948), in which the subcultured bacteria were inoculated in the center of the media and incubated at 28°c for 3 days.

3.6.2. Cellulase activity

Cellulase media was prepared as the protocol which contains 0.2% CMC, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.5%$MgSO_4$, 0.5%KCl, 0.02% Peptone, and 2% Agar. The bacteria was streaked at the center on CMC –Na plates and incubated at 28°c for 24 hours and after incubation, 1% Congo red solution was flooded over the plates to stain the plates for 15 mins and after that 1 M NaCl was used to distain the plates and kept for 15 mins and after that it was poured off.

3.7 Effects of heavy metals on the isolated bacteria

For the analysis of the effects of heavy metals, endophytic bacteria isolated were inoculated on the media supplemented with Cadmium chloride and Mercuric chloride of different concentrations.

Preparation of 1 M $CdCl_2$(stock solution)

1.8 Gms of $CdCl_2$ was dissolved in 10 ml sterile water in sterile falcon tube.

From the stock solution different concentrations: 200, 300, 400, 500,600 (µM) of $CdCl_2$was prepared in NA media.

Preparation of 0.25 M $HgCl_2$ (stock solution)

2.7 Gms of $Hgcl_2$ was dissolved in 40 ml sterile water in sterile falcon tube.

From the stock 20, 30, 40, 50, 60 (µM) concentrations of $Hgcl_2$ was supplied in NA media.

For the bacterial culture, overnight grown broth was taken and optical density (OD) was taken at 620 nm using UV spectrometer. Then the OD was made one (OD=1) using appropriate amount of inoculum and water. It was centrifuged at 4000 rpm for 5 mins and the supernatant was removed. 1 ml of sterile water was added to the pellet and was vortexed for 1 min. Five microliter of bacteria was inoculated into the nutrient agar medium supplemented with different concentrations of $CdCl_2$ and $HgCl_2$.

OD of BBB3 =1.88

For OD 1= 1/1.88=0.532 ml culture + 0.468 ml ADW

3.8 Effects of isolated bacterial endophytes on tomato plants

3.8.1 Seeds sterilization

To observe the effects of bacterial endophytes on tomato plant, first the tomato plant were grown and for this: the tomato seeds from JAI DURGA BEEJ COMPANY were sterilized by the following method. Fifty seeds were first washed with running water for two times and treated with spirit for 5 mins to remove the contaminants from the seeds. Then it was washed with water for three times. The seeds then were treated with Triton –X 100 (Hi - Media) for ten mins and it was washed with distilled water till the foam was clear. Then it was washed with distilled water for three times. After that the seeds were washed with autoclaved sterilized water inside the laminar hood. Then the seeds were washed with 70% ethanol for 15 mins and washed with sterilized water for 4 times. Again the seeds were washed 0.5 % $HgCl_2$ for 10 mins and then washed with sterilized water for 5 times.

3.8.2 Inoculum preparation from the bacteria

Inoculum was prepared similarly like the method mentioned above in section 3.5 and incubated for 48 hours. Then the inoculum prepared at respective incubation periods were applied on the tomato plant weekly for three weeks and as control tomato plant without bacteria was taken.

3.8.3 Seeds Treatment

The seeds were sterilized as described in method 3.8.1 and the sterilized seeds were soaked in inoculum prepared as described in 3.5 for 6 h, the bacterial cultures were diluted in sterilized distilled water to reach the final concentration of 10^6CFU/ml whereas the nutrient broth without bacterial inoculation was used treat the control ones**.**

- Germination percentage
- Root length
- Shoot length

3.8.4 Seedling Treatment

The seeds were sterilized as described above in 3.8.1. Also the agro-pit was prepared in which the dark and light type of soil was mixed in the ratio 2:6 and was autoclaved. Similarly the pots were also sterilized. Then the sterilized seeds were sown into the pot filled with sterilized agro-pit with the help of sterilized forceps. Then the pots were transferred to culture room for germination where the light, dark condition was maintained. After the 15 days the seeds were germinated and they were transferred to another pots with individual seedling and transferred to glass house where the growth condition for plants was maintained.

Comparison was done for:

- Plant height
- Number of leaves
- Number of flowers
- Number of fruits

ON

First treatment (February 22,2018)

Second treatment (March 7,2018)

Third treatment (March 22,2018)

CHAPTER 4. RESULTS

4.1 Isolation and screening of bacterial endophytes

The bacterial endophytes were isolated from the leaf of tomato plant followed by sterilization of leaf sample and culture on nutrient agar plates. About 20 different bacterial colonies were isolated from the leaf.To make sure the bacteria were isolated from the inside of the leaves, the surface sterilization was confirmed to be perfect by touching the surface of the leaves on NA plates, no colonies from the surface were found on the plates , but the colonies were emerged from inside of the leaves (4.1) .

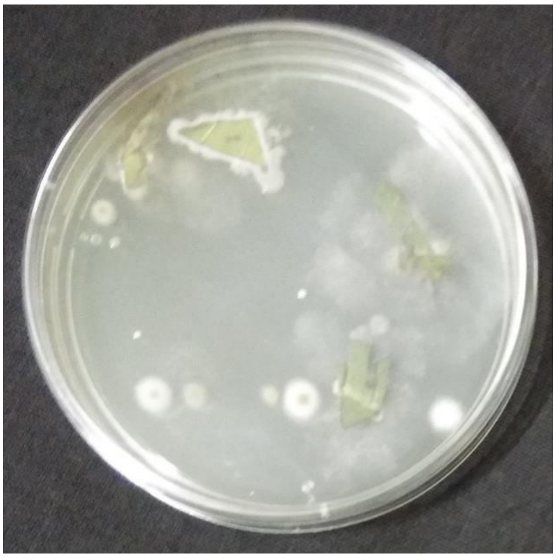


Figure 4.1: Isolation of bacterial endophyte from leaves of tomato plant on NA

Green color with white outgrowth shows the growth of bacterial colonies around the cut leaf

4.2 Preliminary screening of bacterial endophyte against fungi *Alternaria alternata* (At)

The result of the preliminary screening of endophytic bacteria isolated from tomato leaf against fungal pathogen At showed that the isolate BBB3 showed more inhibitory activity on targeted fungi of value 3.4 ±0.4 cm whereas the minimum inhibition was shown by BBB1 of 0.5±0.4 cm.

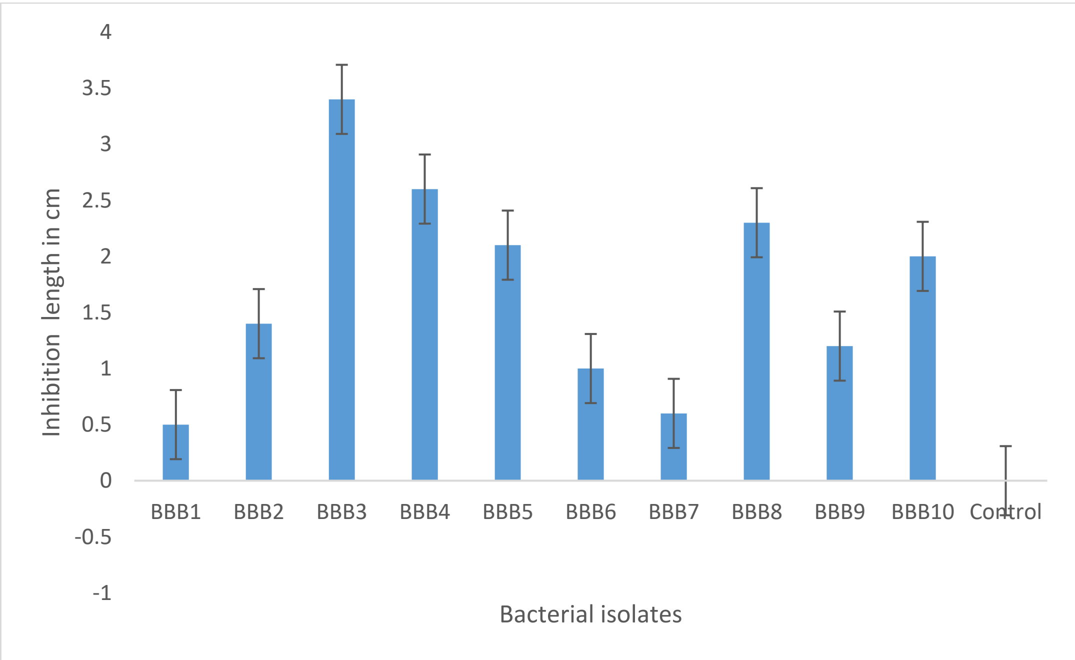


Figure4.2: Preliminary screening of endophytic bacteria against fungal pathogen

Horizontal axis represents the different types of bacterial isolates, Vertical axis represents the inhibition length in cm. The error bars represent the standard deviation from mean value (mean± SD).

4.3 Molecular characterization of bacteria using 16s rRNA sequencing method

The genomic DNA of the isolated bacteria BBB3 had size of 1.5kb when compared with 1 kb ladder. 16S rDNA gene sequence was submitted to GeneBank (http://www.ncbi.nlm.nih.gov).

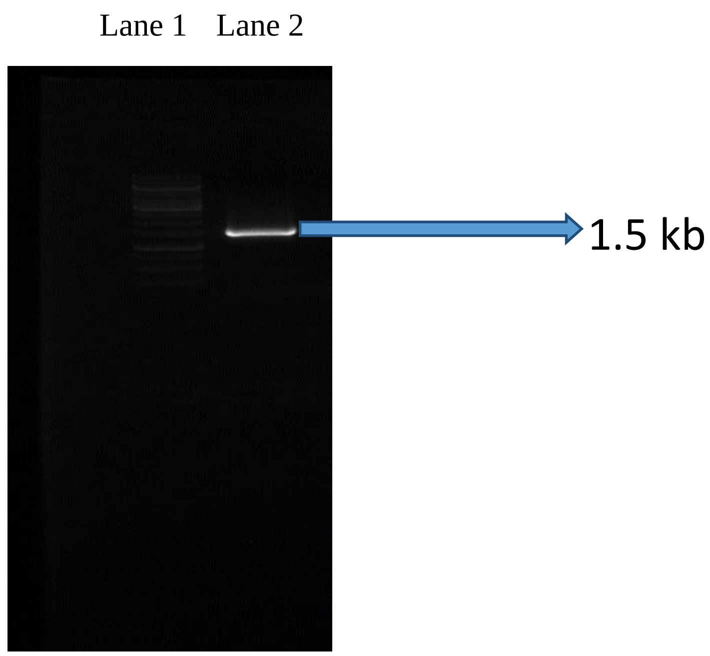


Figure 4.3: Gel electrophoresis (1% agarose) of purified PCR product. (L1 contained 1kb ladder respectively and L2 is the PCR product of the BBB3 respectively.Image was taken from Alpha gel documentation system (Gel Doc)).

4.4 Dual culture assay

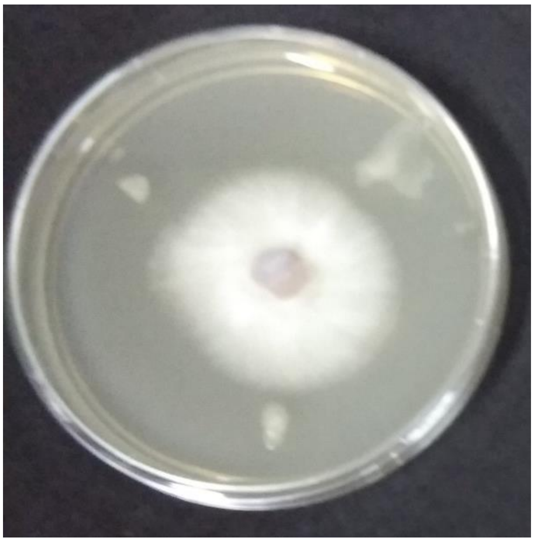
The activity of the isolated bacteria BBB3 was examined against fungal pathogens *Fusarium oxysporum(Fo)* and *Sclerotium rolfsii (Sc)* on PDA plates. At fourth day of incubation the length of the bacteria grown was 4.2 cm and that of *Fo* was 4.8 cm .Similarly the length of bacteria BBB3 with *Sc* was 1.2 and 7.8 cm respectively. On 7 days after incubation the growth of bacteria increased to 4.8 cm while the fungal growth was decreased to 4.2 cm in case of *Fo* and with *Sc, the* case was similar where the growth of bacteria was increased to 1.6 cm and the growth of fungi was ceased to 7.4 cm. The bacteria grew upto 5.3 cm and *Fo* grew upto 3.7 cm after 10 days of incubation and with *Sc* bacteria length was 2.3 cm and of fungi (Sc) was 5.3 cm. The interesting thing was that the control plates were having fungal growth on entire surface of plate which is 9 cm in every above mentioned days of incubation.

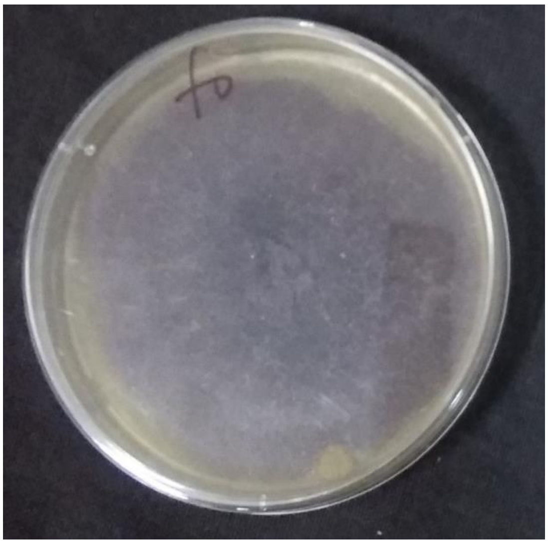
Table 4.4 : Dual culture assay of bacterial endophyte against fungal pathogens

S.N.	**Days of incubation**	**Inhibitory length (cm)**			
		BBB3	***Fo***	**BBB3**	***Sc***
1	4	4.2	4.8	1.2	7.8
2	7	4.8	4.2	1.6	7.4
3	10	5.3	3.7	2.3	5.3
4	Control	0	9	0	9

Fo- Fusarium oxysporum

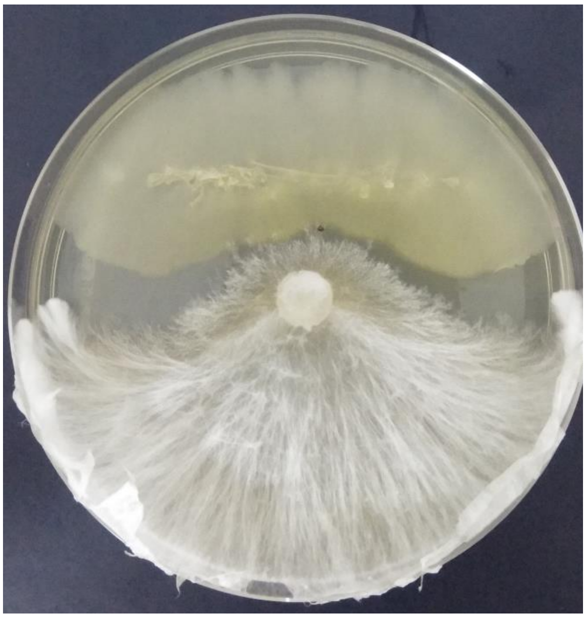
Sc- Sclerotium rolfsii

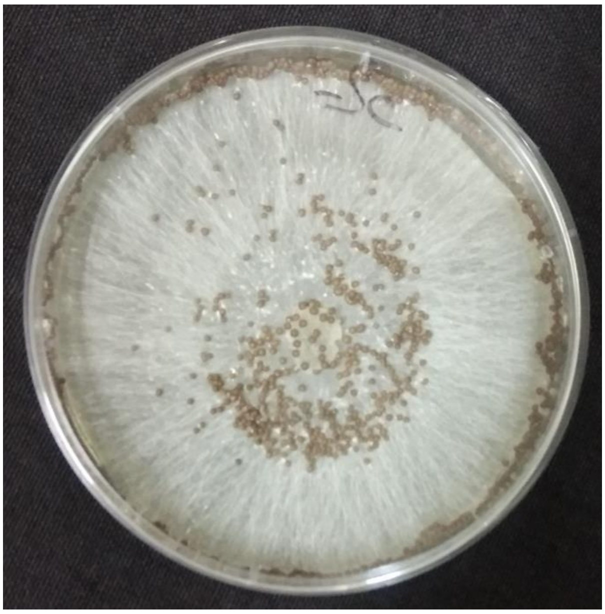




Control Fo

Figure 4.4.1 : Biological control activity using isolated bacteria BBB3 and fungus *Fusarium oxysporum* (Fo)and control is the fungus grown without bacteria





Control (Sc)

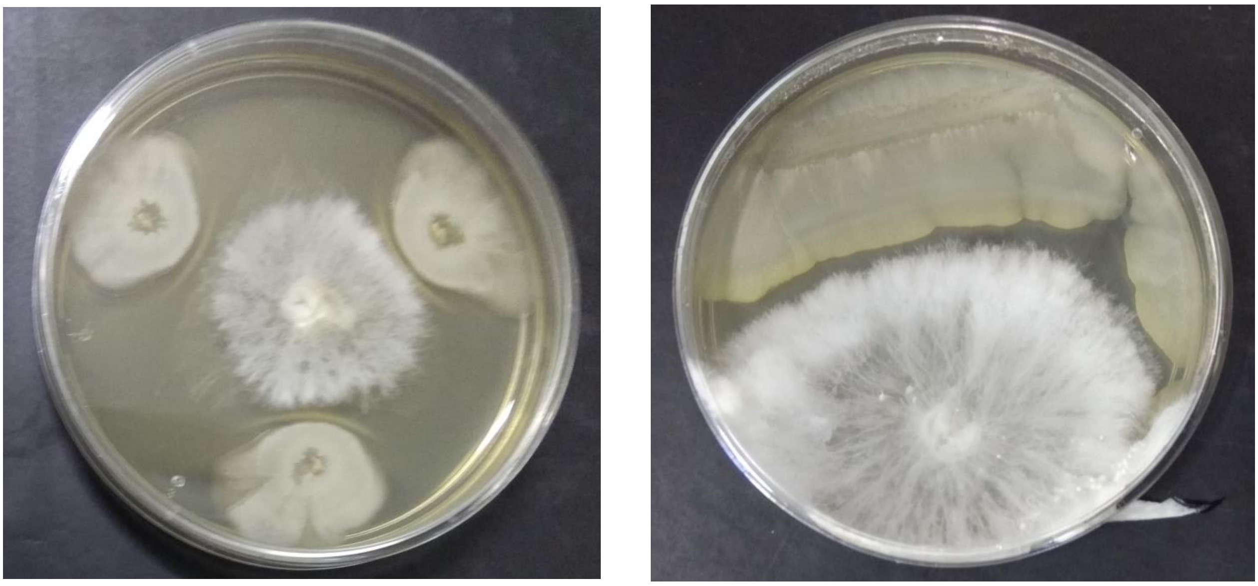


Figure 4.4.2 : Biological control activity using isolated bacteria and fungus *Sclerotium rolfsii* (Sc)

4.5 Liquid Chromatography –Mass Spectrometry for the analysis of metabolites present in the extract

Table 4.5 : LC-MS Profile present in the metabolites of the endophytic bacteria

S.N.	**Name**	**Molecular formula**	**Molecular weight**
1	3-Acetyl-1,2-dithiolane	$C_5H_8OS_2$	148.517
2	Propanoylagmatine	$C_8H_{18}N_4O$	186.1481
3	4-Methoxybenzyl glucoside	$C_{14}H_{20}O_7$	300.1209
4	Pentoxifylline	$C_{13}H_{18}N_4O_3$	278.1379
5	Arachidonoyl Ethanolamide-d8	$C_{22}H_{29}D_8NO_2$	355.7326
6	24-methylcholesta-5,24-dien-3β-ol	$C_{28}H_{46}O$	398.3549
7	Grayanotoxin I	$C_{22}H_{36}O_7$	412.861
8	9,14,19,19,19-pentadeuterio-1α,25-dihydroxyprevitamin D3 / 9,14,19,19,19-pentadeuterio-1α,25-dihydroxyprecholecalciferol	$C_{27}H_{39}D_5O_3$	422.5599
9	Cycloprotobuxine C	$C_{27}H_{48}N_2$	400.3817

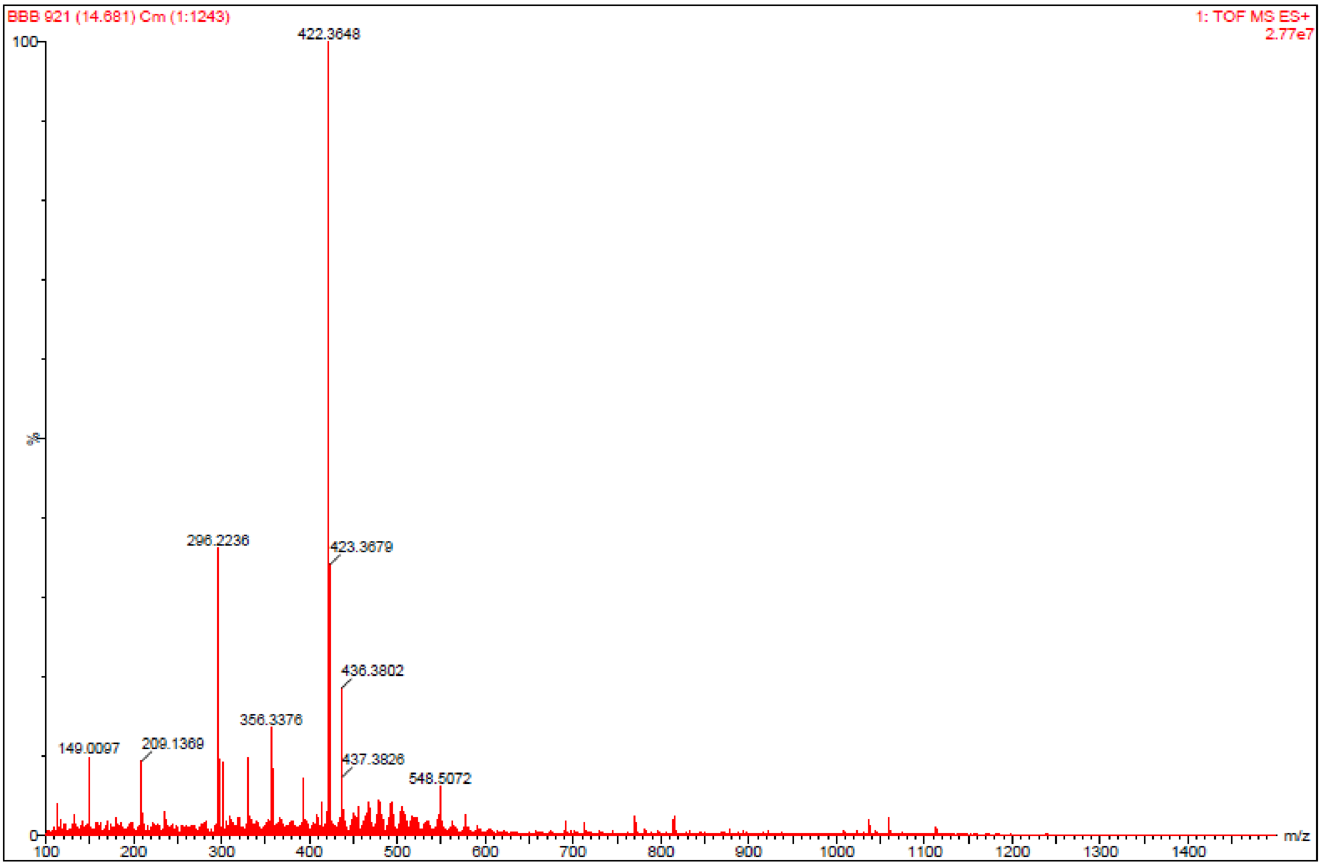


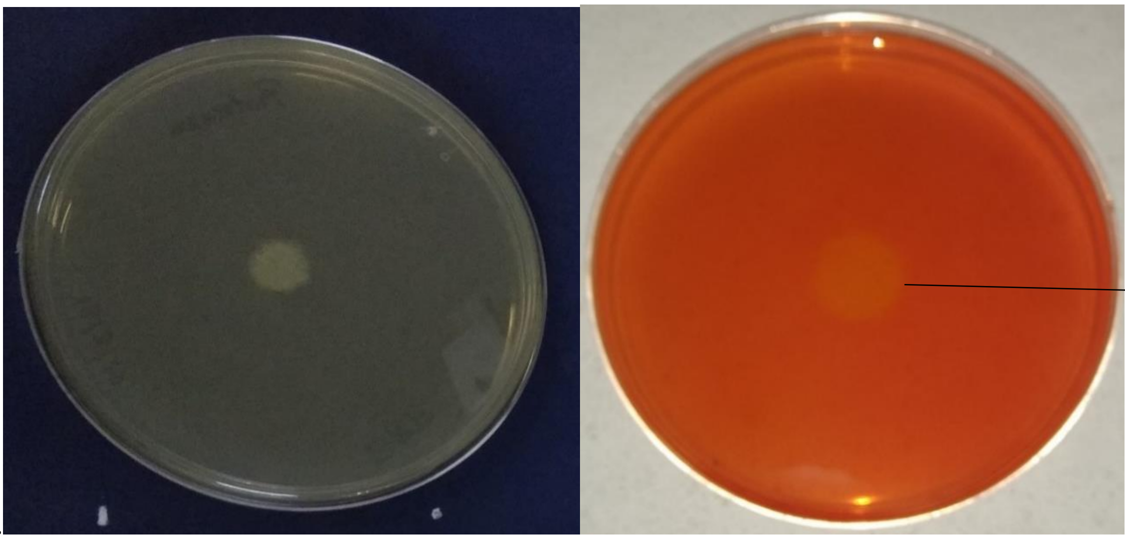
Figure 4.5: TOF-MS spectra of the biological active metabolites responsible for the antimicrobial activity of the endophytic bacteria

4.6 Enzyme activity of the isolated bacterial endophyte

The isolated bacteria BBB3 was able to show some enzymatic activities which is related directly or indirectly to plants.

Cellulase activity

The bacteria on CMC media after staining and formed a clear(halo) zone around the colonies which produced cellulase enzyme.



a. b.

Figure4.5.1:Cellulase activity

a: Bacteria in CMC agar plates before staining

b: Bacteria after staining forming halo zone

Phosphate solubilizing activity

When the isolated bacteria was grown on Pikovskayas agar medium ,after incubation for 3 days at 28°C ,clear zone was visible around the colony which confirms that the bacteria showed the phosphate solubilizing ability by utilizing the insoluble tricalcium phosphate present in the medium.

Figure4.5.2: Phosphate solubilization activity

4.6 Effects of heavy metals on the growth of isolated endophytic bacteria

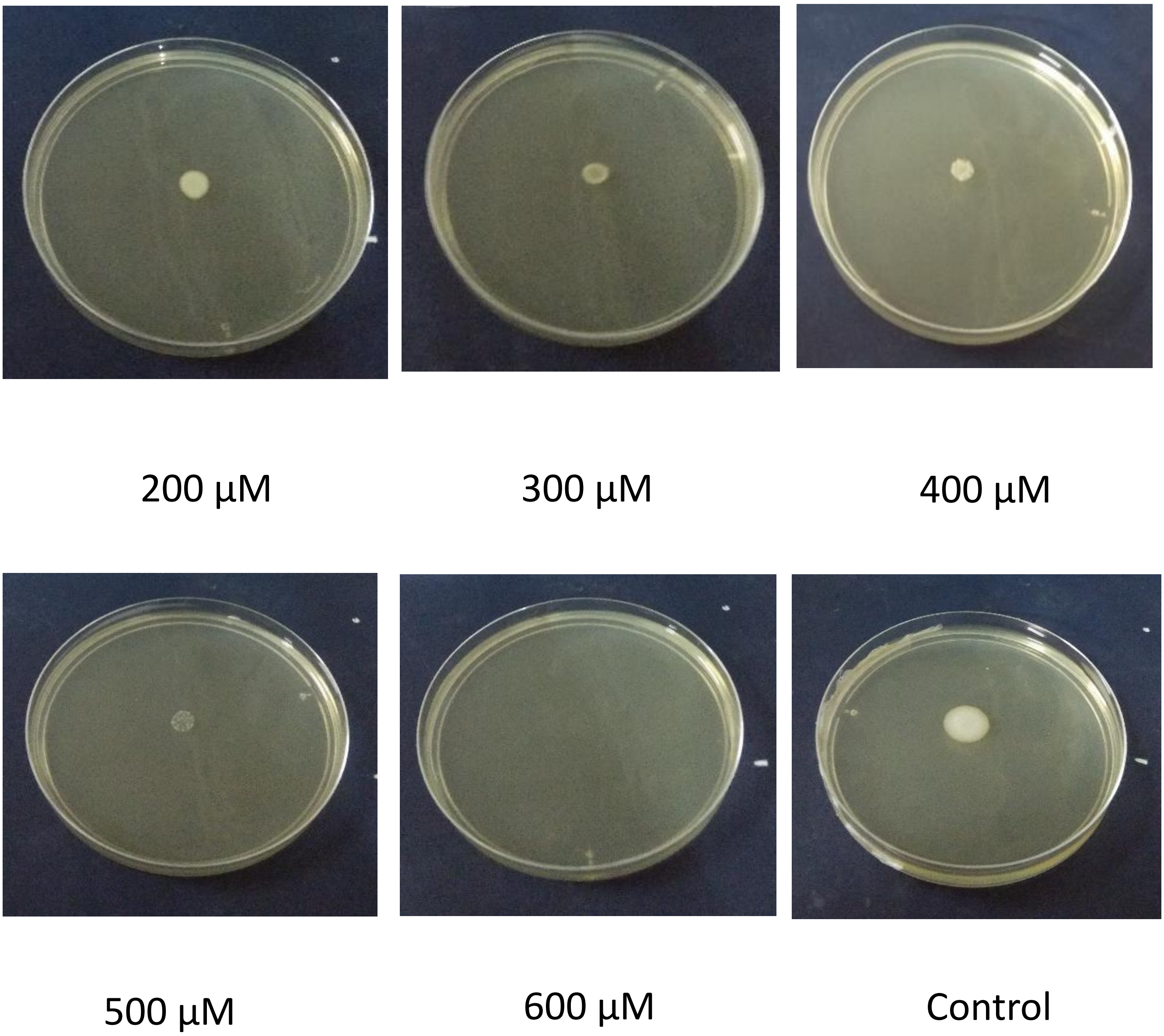
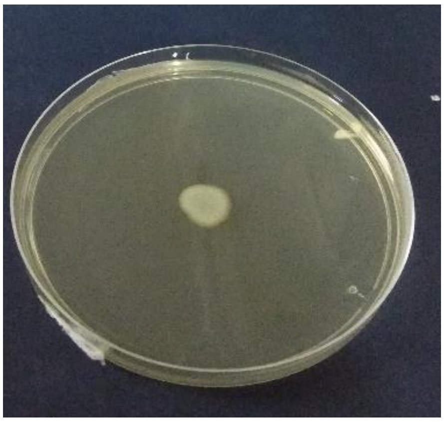
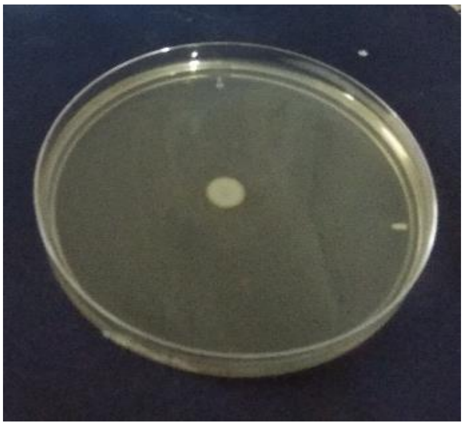


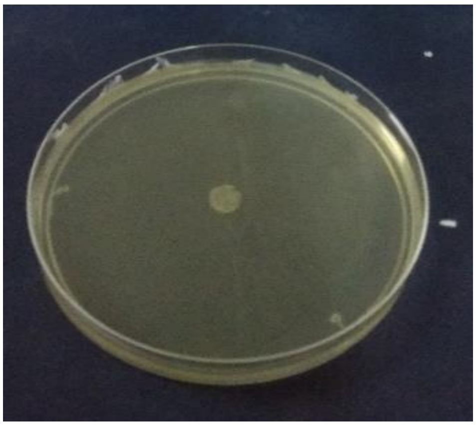
Figure 4.6.1: Different concentration of Cadmium chloride and bacterial growth



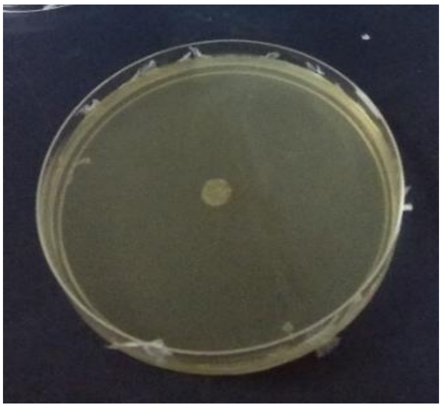
20 µM



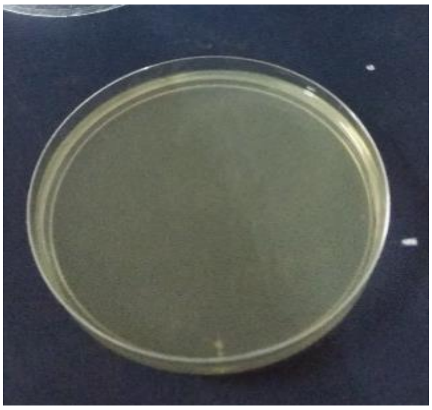
30 µM



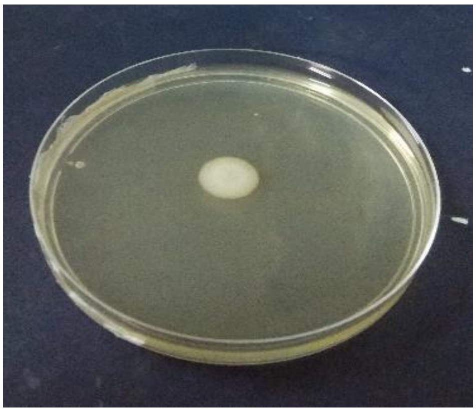
40 µM



50 µM



60 µM



Control

Figure 4.6.2 : Different concentration of Mercuric chloride and bacterial growth

Table4.6: showing the the concentration heavy metals and growth of bacteria

S.N	Concentration of Cadmiumchloride (µM)	Bacterial growth	Concentration of Mercuric chloride(µM)	Bacterial growth
1	200	+	20	+
2	300	+	30	+
3	400	+	40	+
4	500	+	50	+
5	600	-	60	-

Note : "+"denotes that the presence of bacteria on the plates containing heavy metals

"-" denotes the absence of bacteria on the plates

4.7 Germination index

The tomato seeds were visualized for their germination using bacterial inoculum (treated) and without using inoculum (control) at equal interval of time after sow. This experiment was performed for one set of plants only.

Table 4.7: Germination percentage of treated and control tomato seeds

S.N	Type of seeds	Germination number	Germination percentage (%)
1	Treated with BBB3	15	75
2	Treated with BBB4	13	65
3	Treated with BBB8	9	45
4	control	5	25

4.8 Measurement of different parameters of tomato plants

4.8.1 Seeds treatment

The seeds of tomato after sterilization and inoculum of isolated bacteria was given to one set while another set was not given were germinated. The plant was studied for the parameters after equal day of inoculation. When the different parameters of tomato plant were analyzed, the seeds that were treated with isolated endophytic bacteria BBB3 were having improved parameters as compared to other treated (BBB4, BBB8) and untreated ones. The root length of seeds treated with BBB3 was 8.1±0.07cm whereas control was 4.2±0.42 cm. The shoot length was found to be 14±0.42cm of the seeds treated with BBB3 whereas of control was7.3±0.14cm. The value of leaves number of treated was 19±1.41, whereas of control was 7±1.41. The error bars are less significant because there can be variation in the biological parameters.

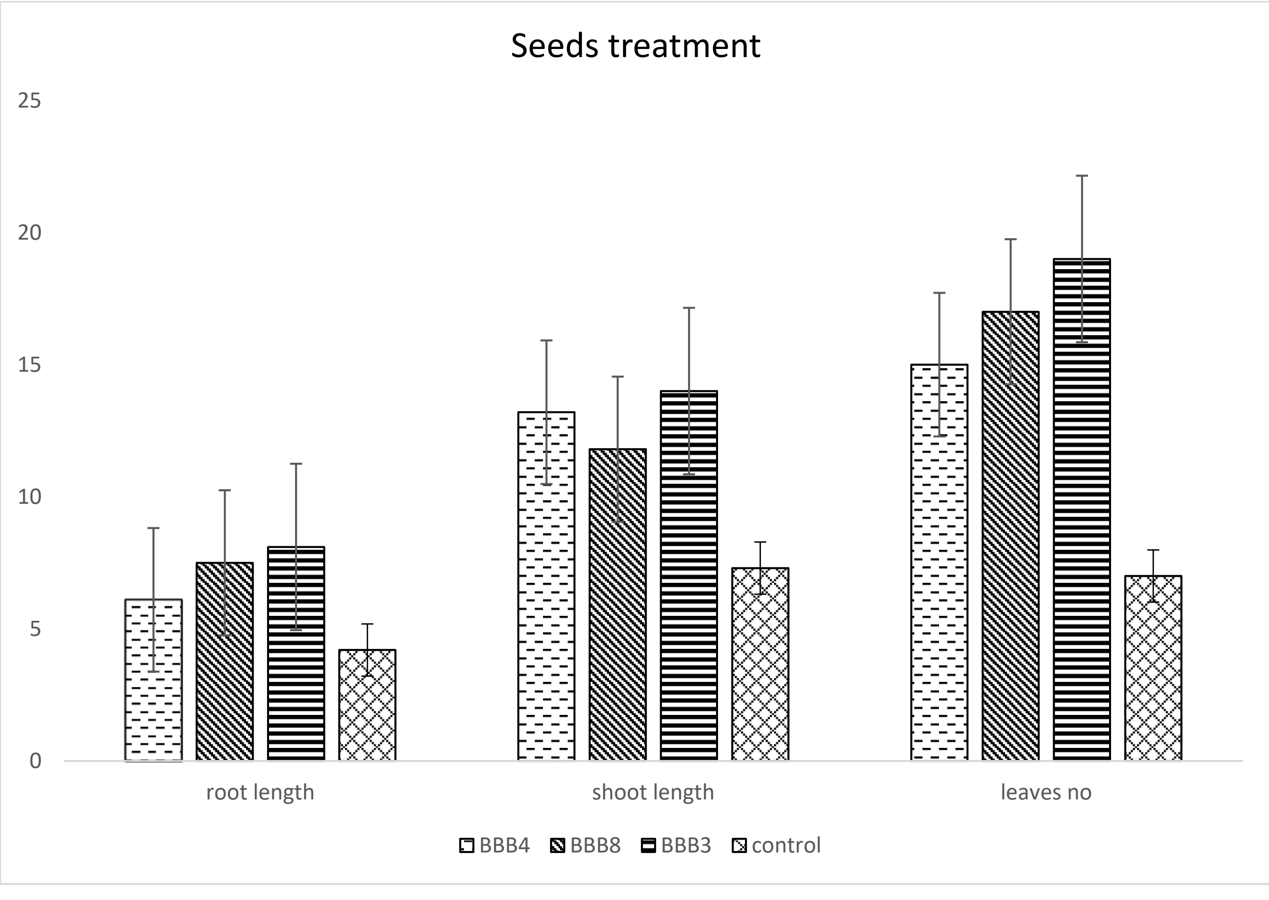


Figure4.8.1 : Seeds treatment and measurement of different parameters of tomato plant

Tomato seeds treated with isolated endophytic bacteria BBB3, BBB4, BBB8

Control denotes tomato seeds without giving inoculum

(Error bars denotes the average of three values±SD), P≤0.05

4.8.2 Seedling treatment

First treatment

When the different parameters of tomato plant were analyzed, the plants that were treated with isolated endophytic bacteria BBB3 were having improved parameters as compared to untreated ones. The value of height of the treated was 31.6±1.345cm whereas control was 28.4±2.107 cm. The value of leaves no of treated was 49.6±1.527, whereas of control was 32.6±2.08.

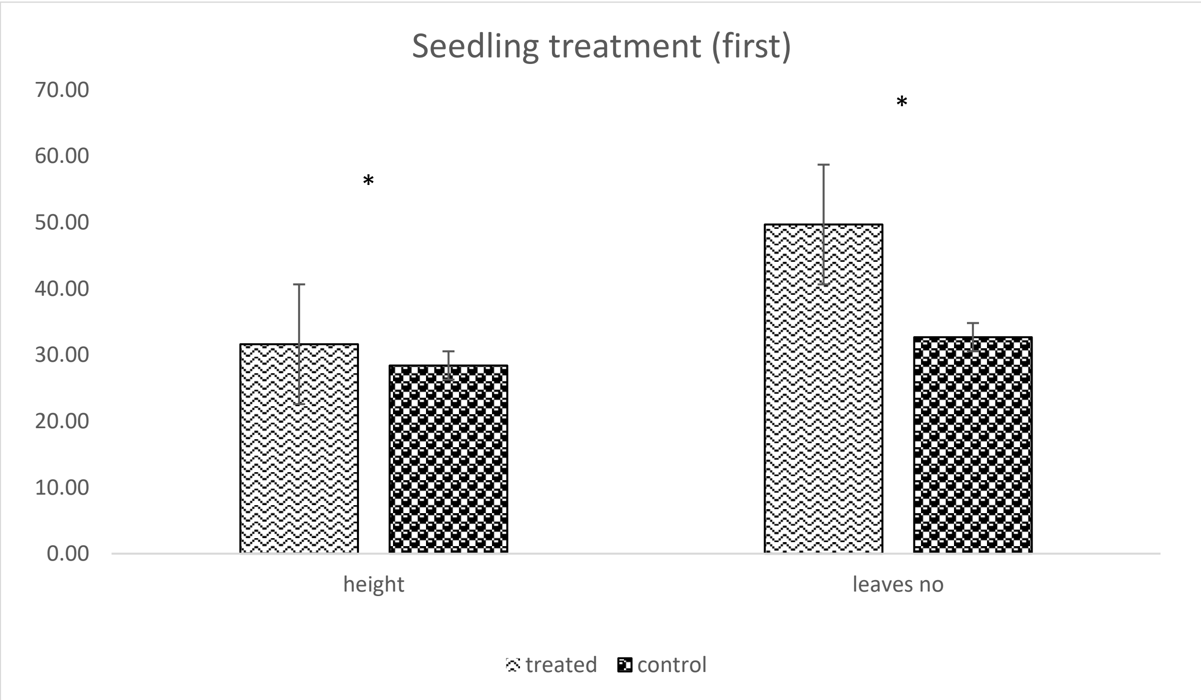


Figure4.8.2 :Seedling treatment and plant parameters (first treatment). The error bars are represented as mean ±SD where, P ≤0.05

Second treatment

When the different parameters of tomato plant were analyzed, the plants that were treated with isolated endophytic bacteria BBB3 were having improved parameters as compared to untreated ones. The value of height of the treated was 39.2±0.802 cm whereas control was 35.3.5±0.964 cm. The value of leaves no of treated was 50.6±3.214, whereas of control was 38.0±3.605. The value of flower number of treated was 3±1.732 whereas of control was 1.3±0.57.

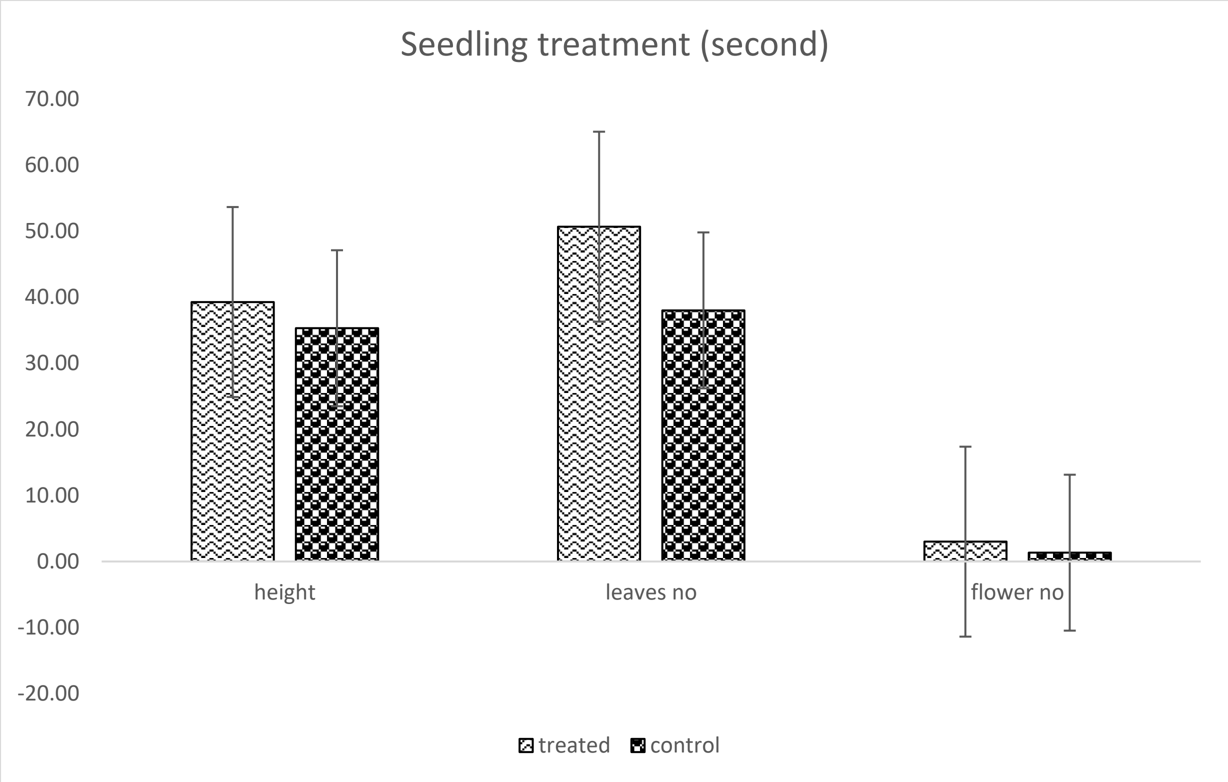


Figure 4.8.3 : Seedling treatment and plant parameters (second treatment). The error bars are represented as mean ±SD, P ≤0.05

Third treatment

When the different parameters of tomato plant were analyzed, the plants that were treated with isolated endophytic bacteria BBB3 were having improved parameters as compared to untreated ones. The value of height of the treated was 42.0±1.25cm whereas control was 33.5±0.90 cm. The value of leaves no of treated was 54.6±3.05, whereas of control was 36.3±3.05. The value of flower number of treated was 3±1.00 whereas of control was 1.3±0.57. The last parameter that is the fruit number showed average value of 2.6±0.57 in treated whereas no flower in control plants. The error bars are less significant because there can be variation in the biological parameters.

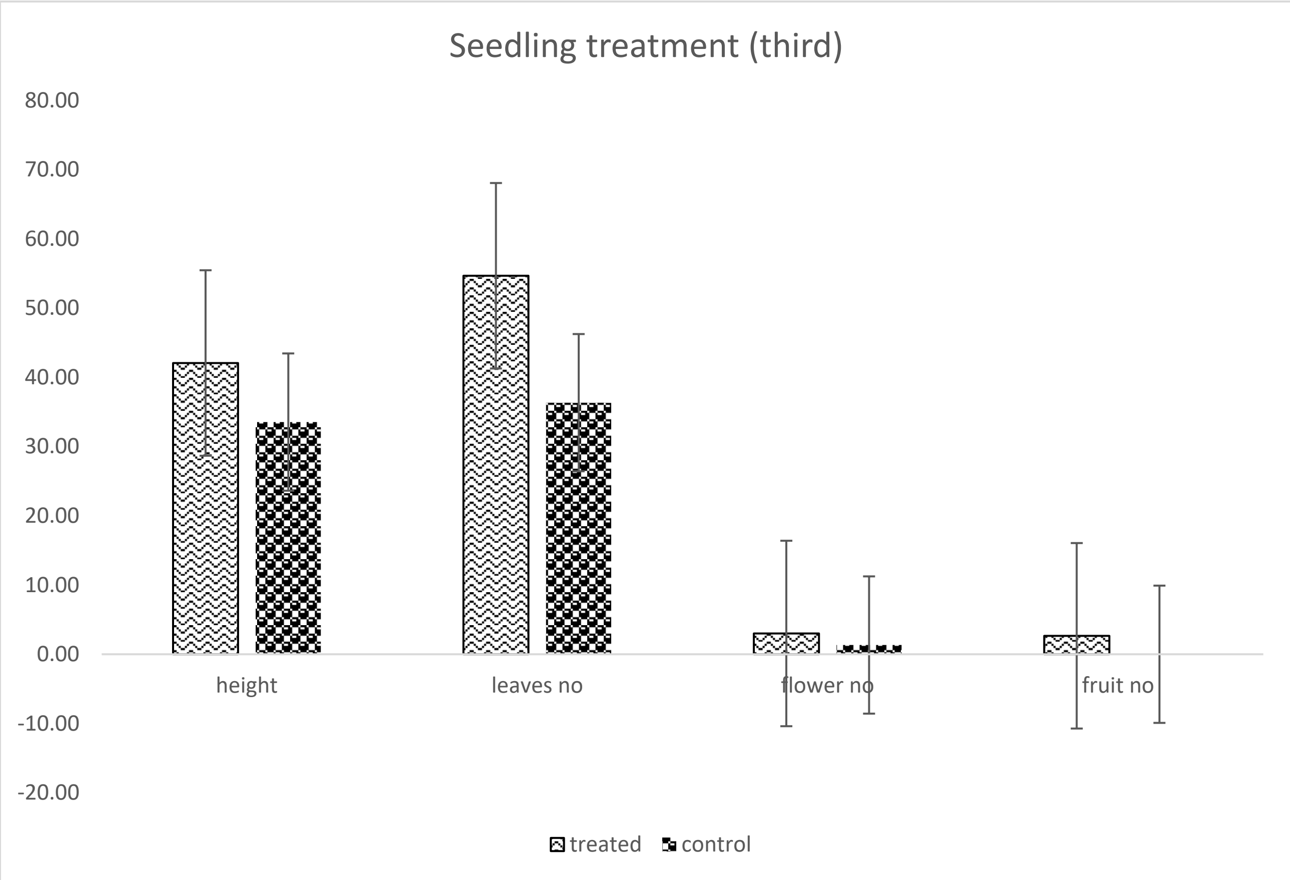


Figure 4.8.4 : Seedling treatment and plant parameters (third treatment)Treated denotes tomato plants treated with isolated endophytic bacteria BBB3Control denotes tomato plant without giving inoculum(Error bars denotes the average of three values±SD), P≤0.05

CHAPTER 5. DISCUSSION

Tomato is a major vegetable crop that has achieved tremendous popularity over the last century. It is grown in practically every country of the world - in outdoor fields, greenhouses and net houses.

Also it is one of the most popular vegetables worldwide which is used in either cooked form or simply as raw (salad). But there are many limiting factors for its cultivation out of which infection is one of the major cause. This will decrease the yield and ultimately hamper the overall productivity. The pathogens responsible for this are bacteria, fungi, nematodes, virus, and insects and so on. Jones et al., (1991) presented major diseases of tomato caused by 24 fungi, 7 bacteria, 10 viruses, 3 viroids, and multiple nematodes.

It is known that the horticultural crops are facing the problem of many phyto- pathogenic bacteria and fungi which cause plant diseases and the chemical controlling agents that is, fungicides and fumigants are restricted in high amount. So, as an alternative method for disease suppression and control, microorganisms found inside the plants are carrying major importance as given by (Burkholderia et al., 2011). However different practices have been done to avoid chemical pesticides from local as well as government level in the context of country like Nepal.

Endophytes are the microorganisms that are found inside the plant body either intercellularly or intracellularly as a part or for whole life cycle. They generally include bacteria, fungi, actinomycetes and so on. They are inhabitant of plant tissues without causing harm and are distributed in various parts of plant body such as: leaves, twigs, stem, petioles fruit, flower, root etc.. The isolation and determination of plant related endophytes could be a boon to biotechnological applications as biological control agents or plant growth promoters (Hassan, 2017).

5.1 Isolation of bacterial endophytes

Twenty different bacterial colonies were isolated from the leaf by surface sterilization and culture techniques. As a result of preliminary screening against fungal pathogen *Alternaria alternata*, BBB3 showed the highest inhibition so, it was chosen for further analysis.

5.2 Biological control activity against fungal pathogens

The isolated bacteria was tested against tomato pathogens (fungal pathogens): *Sclerotium rolfsii* and *Fusarium oxysporum*. The bacteria was able to inhibit them, acting as biological control agents. The ability to inhibit the fungal pathogens was persistent upto ten days of inoculation. As BBB3 was able to suppress *Sclerotonia rolfsii* and *Fusarium oxysporum*, these are also the major pathogens resulting the loss of economically important agricultural crops .The inhibiotory activity of bacteria against the fungal pathogens suggests the use as effective biological control agents (Ohike et al., 2013). The size of bacteria seemed to be increasing due to the extract produced by the endophytic bacteria on long duration of incubation.

5.3 LCMS profile of the biological etract of bacterial endophyte BBB3

According to LC-MS analyses, all compounds detected in the endophytic bacteria have their pharmaceutical and medicinal use and reported as anti-inflammatory, anthelmintic, antibacterial and antifungal compounds. Antifungal activities detected in the strains are due to production of these bioactive metabolites secreted by antagonistic endophytic bacterial strains. The compound identified as secosteroid (9,14,19,19,19-pentadeuterio-1α,25-dihydroxyprevitamin D3 / 9,14,19,19,19-pentadeuterio-1α,25-dihydroxyprecholecalciferol) is found to have major role in human being during pregnancy and also in controlling calcium and phosphorus homeostasis (Perez-lopez, 2014).

5.4 Enzyme activity

5.4.1 Cellulase activity

The isolated bacteria BBB3 showed cellulase activity by forming a halo zone on the media plate containing CMC. It was qualitative assay which signifies that this bacteria might have penetrated into the plant body by this activity because cellulose is one of the compound found majorly on plant cell wall. The hydrolytic enzyme like cellulase is responsible to assist plant by aiding endophytes to enter the plant tissues and also protects the plant by invading the pathogenic microorganisms thorough hydrolysis of cell wall (Choi et al., 2005). This result also support of introducing cellulose degrading bacteria as useful technique for energy recovery from degraded ecosystem. Also the isolated bacteria showing the cellulase activity signifies the presence of cellulose decomposer which can break down large organic compounds into suitable forms that can be utilized by the biotic community. With the help of microorganisms ultimately the source for the utilization of renewable source of energy becomes possible which is a boon in the field of ecology and microbiology (Kaur & Arora, 2012).

5.4.2 Phosphate solubilization activity

Phosphate solubilization by microorganisms is another benefit provided by endophyte as bio fertilizers and the isolated bacteria BBB3 is showing halo zone on media provided with phosphate which indicates that the bacteria has potent to solubilize phosphate which ultimately contribute to plant growth and development. There are different mechanisms about how bacteria become able to solubilize insoluble phosphate, one is by secretion of organic acids and another is through the excretion of protons or enzymatic production that helps to make insoluble phosphate available to plant by mineralizing organic phosphate (Hassan, 2017).

5.5 Effects of heavy metals (Cadmium and Mercury)

The isolated endophytic bacteria BBB3 has grown on the concentration from 200 to 500 µM of Cadmium chloride (a heavy metal) but the growth is inhibited at a concentration of 600 µM. It shows that the bacteria can tolerate a $CdCl_2$ concentration upto 500 µM which is a very high amount. The isolated endophytic bacteria BBB3 has grown on the concentration from 20 to 50 µM of Mercuric chloride (a heavy metal) but the growth is inhibited at a concentration of 60 µM. It shows that the bacteria can tolerate a $HgCl_2$ concentration upto 50 µM which is a very high amount. Due to the effects of heavy metals, a worldwide problem of soil contamination is affecting the agriculture and also the health of many people via food chain (Ma et al., 2011). So as a solution strategy remediation techniques should be implemented and using plant and microbe is one of the effective way to solve this global issue via phytoremediation.

5.6 Green house assay on tomato seeds and seedlings

The seeds inoculated with isolated bacteria were able to germinate faster as compared to control at the equal interval of sow which suggests that endophytes can help plants to germinate at a faster rate which ultimately decreases the overall time for crop harvest.

In green house experiment the inoculated seeds showed the high germination percentage as compared to uninoculated one. Also the different plant parameters of root length, shoot length of the inoculated seeds were better as compared to the uninoculated seeds. Also the tomato seedlings were compared for height, leaves number, fruit number, and flower number in green house providing similar condition with the seedlings that were not inoculated with isolated bacteria BBB3. The result showed the improved parameters in the inoculated ones. The performance of an endophyte can affected by various factors such as, nitrogen present in the soil, availability of water, soil type (sterile or non- sterile) and texture (Botta et al., 2013).

Table5.7: showing the comparison of plant parameters between BBB3 and control

S.N	Seed treatment	Result
1	Germination	BBB3> control
2	Other parameters(RL,SL,leaf no.)	BBB3> control

S.N	Seedling treatment	First treatment	Second treatment	Third treatment
1	Parameters (PH,LN,FloN,Fr N)	BBB3>control	BBB3>control	BBB3>control

“>”Denotes showing better activity

- RL=Root length
- SL=Shoot length
- PH=Plant height
- LN=Leaf number
- FloN=Flower number
- FRN= Fruit number

CHAPTER 6. SUMMARY

Hence endophytic bacteria was isolated using tomato as a model plant and its biological control activities were assayed against the fungal pathogens of tomato plant where the isolated bacteria BBB3 showed inhibition upto ten days of inoculation. The bacteria was characterized using 16s rRNA sequencing method and the bacteria was subjected to other tests where it proved itself to be involved in plant growth promoting activities. The LCMS result suggested that the bioactive compounds produced by the bacteria can aid for the natural defense mechanisms. The bacteria showed cellulase and phosphatase enzyme activities. Then another test was done using heavy metals (Cadmium chloride and Mercuric chloride) where the isolated bacteria was found to be surviving at a high concentration of Cadmium (upto 500 µM) and Mercury (50 µM) which directs its use in phytoremediation. Then the inoculants of the isolated bacteria was analyzed in green house using tomato seeds and seedlings where the bacteria treated one showed the improved parameters of high germination index, increased number of leaves, fruits, flowers, root length, shoot length, plant overall height and so on as compared to untreated one which showed its (endophytic bacteria BBB3) involvement in plant growth promoting activities.

CHAPTER 7. CONCLUSION

Endophytes are the microorganisms that reside inside the plant tissues without causing any harmful effects. They are found to support plants for growth and development by various mechanisms: by aiding the plant for nutrient uptake (phosphorus, iron etc). They help in plant defense mechanisms by controlling the plant pathogens in the form of biological control agents. They are also reported to help to develop systemic resistance against the plant pathogens.

In the present investigation bacterial endophyte BBB3 was isolated from the leaf of tomato by general sterilization technique which concludes that leaf is one part of the plant which harbor the endophytes. Simple technique of sterilization can be useful to isolate different types of microorganisms. The isolated bacteria showed anti-fungal activity which directs to the use of endophytes as biological control agents. Biological control agents can be a better alternate to the use of chemical fertilizers which is the ultimate need of today's world as a solution to global problem of pollution. Such a small organism (bacteria) is capable of producing bioactive compounds which help plant to invade the pathogens and also carry importance for the production of pharmaceuticals as well is concluded frpm the LCMS result. The bacteria was able to show different enzyme activities like cellulase, phosphate solubilization which signifies the use of these bacteria in plant growth promoting activities. Also these enzyme activities can be directed to other era of industrialization by mass production from these bacteria. The bacteria is also surviving at high concentration of heavy metals that means it has capacity to resist the effect of heavy metals which can be applied for plants also to aid in phytoremediation. In order to enhance the agricultural yield bacterial inoculation is one of the efficient way. Bio-inoculation is a promising measure to sustainable practice in agriculture, because microorganisms establish associations with plants and promote growth. Here in our study the bacteria residing inside the tomato plant is helping tomato plant (host) for growth and development. It is somehow protecting the plant from the pathogens and effect of heavy metals also which sounds to be very interesting which the overall conclusion of the research.

Thus, the introduction of beneficial bacteria in the soil sounds to be less aggressive and cause less harmful impacts to the environment than chemical fertilizers which makes a sustainable agricultural practice.

CHAPTER 8. RECOMMENDATIONS

From this preliminary research I would like to make the following recommendations.

- The number of strains and species of endophytes vary from plant to plant and the exploration of their role depend on the intensity of study and research so there is still a long way to unravel the world and function of endophytes.
- The extract from the endophytes could be another subject of study. Further analysis must be done using different techniques such as chromatographic techniques (gas chromatography, liquid chromatography), spectrometry techniques and so on in order to attain information about the composition of the extract.
- Metabolomics is another technique that is recommended in order to understand the end metabolites that are playing a major role in providing the endophyte the peculiar function.
- Nanoparticles synthesis using the endophytic extract is also recommended as agriculture is the major concerned subject for a country and if the endophytes are made available in better options like nanoparticles, their value will be added more.

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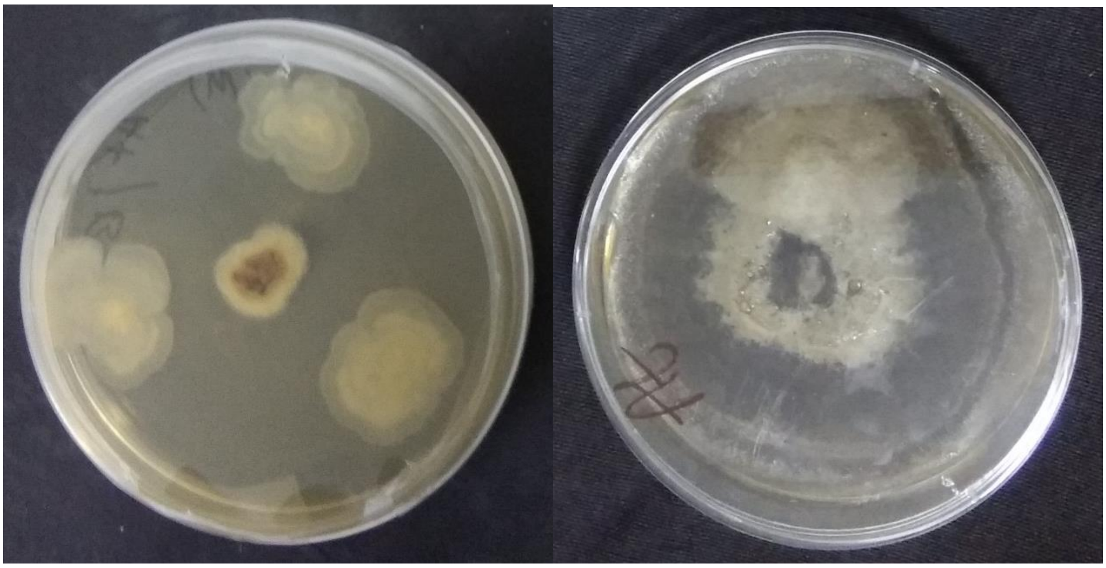
APPENDIX

Figure A.9.1 : Sterilization of Leaf of tomato



Control

Figure A.9.2 : Biological control activity using isolated bacteria BBB3 and fungus *Alternaria alternata* (At) and control is the fungus grown without bacteria

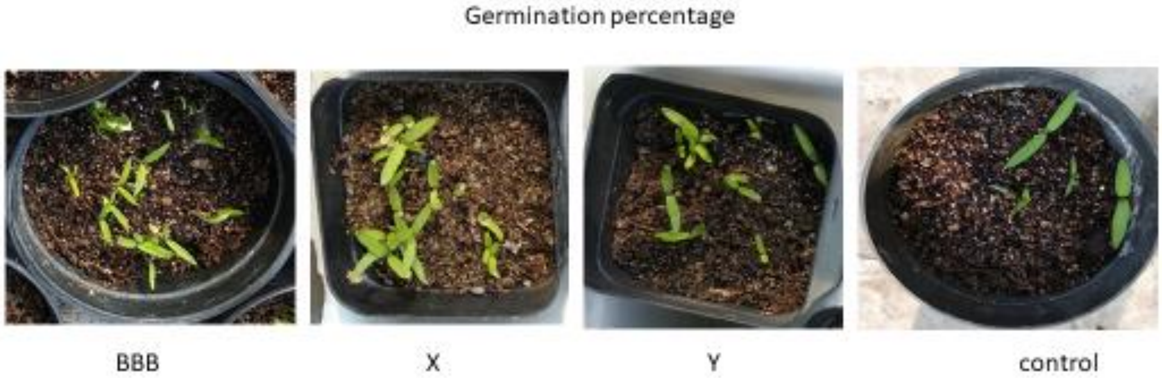


Figure A.9.3 : Germination percentage of seeds (inoculated and not inoculated with isolated bacteria BBB3)

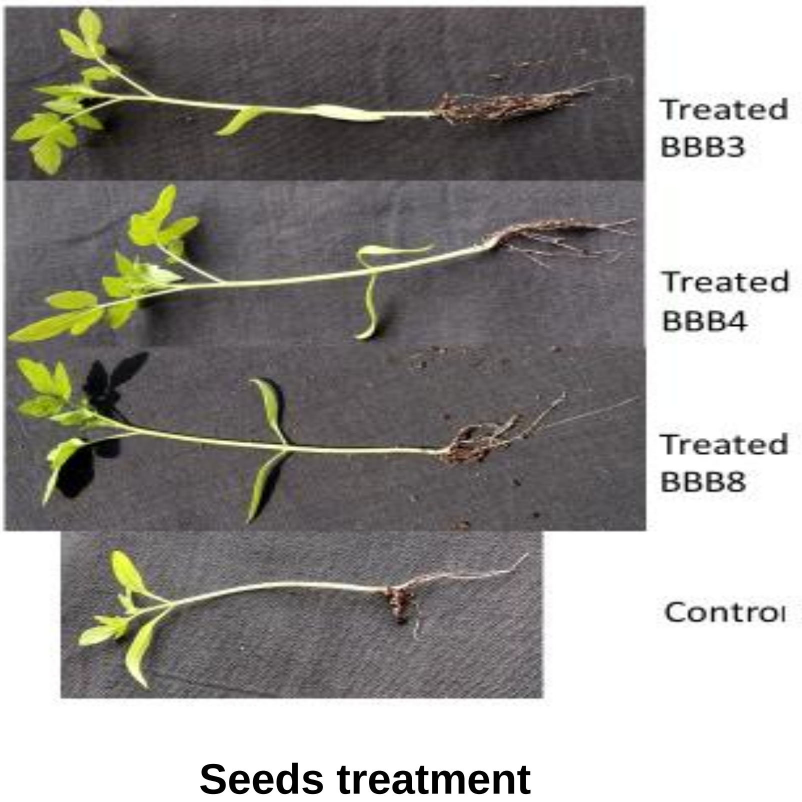


Figure A.9.4 : Treatment of seeds with isolated bacteria and analysis of the different growth parameters between treated and untreated one(control)

Treated Control

Figure A.9.5 : Seedling treatment (first) and analysis of different plant parameters

Treated Control

Figure A.9.6 :Seedling treatment (second) and analysis of plant parameters

Figure A.9.7 :Seedling treatment (third) and comparison of different plant parameters between treated and control one

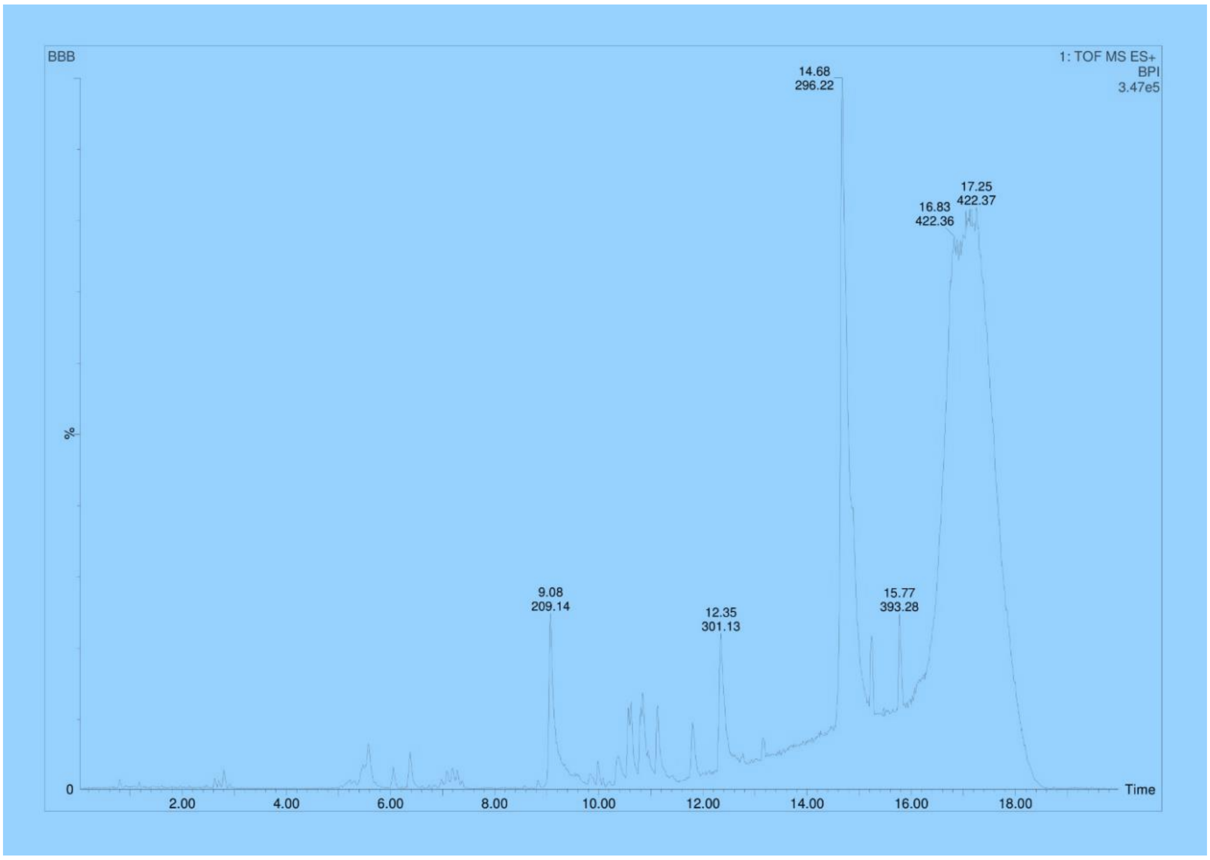


Figure A.9.8 : Mass spectrum of the BBB3 extract by LCMS

Figure A.9.9 : Some participations during the thesis work

Table A.9.1 : Composition of Nutrient Agar media

The composition of nutrient agar media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follows:

S.N	Components	Grams/L
1	Peptic digest of animal tissue	5.00
2	Beef extract	1.50
3	Yeast extract	1.50
4	Sodium chloride	5.00
5	Agar	15.00
6	pH	7.4± 0.2

Dissolve 13.0 grams in 1000 ml of distilled water.Heat to boiling to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 mins. Cool the media and pour into sterilized petriplates

Table A.9.2 : Composition of Luria Bertani Broth, (LB) Miller media

The composition of Luria Bertani broth, (LB) Miller media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follows:

S.N	Components	Grams/L
1	Casein enzymic hydrolysate	10.00
2	Yeast extract	5.00
3	Sodium chloride	10.00
4	pH	7.5± 0.2

Dissolve 25.0 grams in 1000 ml of distilled water.Heat to boiling to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 mins. Cool the media and pour into sterilized petriplates

Table A.9.3 : Composition of Potato Dextrose Agar media

The composition of potato dextrose agar (PDA) media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follows:

S.N	Components	Grams/L
1	Potatoes infusion	200.00
2	Dextrose(Glucose)	20.00
3	Agar	15.00
4	pH	5.6±0.2

Dissolve 39.0 grams in 1000 ml of distilled water.Heat to boiling to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 mins. Cool the media and pour into sterilized petriplates.

Table A.9.4 : Composition of Pikovskaya's Agar media

The composition of Pikovskaya's agar (PVK) media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follows:

S.N	Components	Grams/L
1	Yeast extract	0.5
2	Dextrose	10.0
3	Calcium phosphate	5.0
4	Ammonium sulphate	0.5
5	Potassium chloride	0.2
6	Magnesium sulphate	0.1
7	Manganese sulphate	0.0001
8	Ferrous sulphate	0.0001
9	Agar	15

Dissolve 31.3 grams in 1000 ml of distilled water.Heat to boiling to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool the media and pour into sterilized petriplates.